BIOCHEMICAL STUDIES ON HEMATOPOIETIC AGENTS

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PREFACE

One of the necessary fundamental approaches to an understanding of malignant growth is the study of normal growth - the orderly proliferation and differentiation of cells. After we had considered a number of possible studies which might be undertaken the objectives of which were thought to contribute to an understanding of cancer, it was decided to undertake investigation of some of the chemical agents and biochemical processes incident to the pathological behavior of the bone marrow in pernicious anemia. In pernicious anemia the bone marrow resembles in many respects malignant tumors; the amount of tissue increases but it becomes functionally inefficient. The bone marrow cells which would normally develop into the red blood cells multiply at an exaggerated rate but remain in a primitive form and never mature. The conversion of the tissue from this pathological condition to a normal state had been shown to be effected by any one of several specific substances, (a) an erythrocyte maturation factor, (b) an incubation mixture of an "extrinsic factor" and the "intrinsic factor", (c) folic acid and (d) thymine. These materials, then, are intimately involved in establishing the conditions necessary for the normal functioning of at least one type of tissue.

Consequently, an extensive and comprehensive research of the literature was made in order to collect together and correlate the known physiological and chemical aspects of pernicious anemia (and morphologically related disorders, (with the chemical agents and biological factors which had been shown to be involved. A summary of this literature research constitutes the first section of this thesis.

During the assembling of this material there became evident a number of interesting hypotheses which might explain some of the isolated observations which had been reported. Several of these hypotheses were tested for their validity and the summary of this experimental work constitutes the second portion of this dissertation.

SECTION I

BACKGROUND

A.

BIOLOGICAL CHANGES ASSOCIATED WITH PERNICIOUS ANEMIA

Morphological Changes. Most of the pathological conditions involved in pernicious anemia are associated with distinctive morphological changes that occur in (a) the peripheral blood system, (b) the bone marrow, (c) the mucosa of the gastro-intestinal tract, and (d) the nervous system.

The characteristic alteration in the peripheral blood system is usually the symptom first recognized and is the reason for classifying the disease as an anemia. This particular anemia is of the macrocytic, hyperchromic type - a type in which the reduction in the total number of red blood cells is partially offset by an increase in the size and hemoglobin content of the individual cells (macrocytosis). In general the number of macrocytes can be correlated with the severity of the anemia. Bizarre shaped cells can usually be noted, though they frequently develop only when the disease has reached an advanced stage. The hemoglobin content of the cells increases in proportion to the increase in cell size; but there is no hemoglobin "super-saturation" as is sometimes implied. The "blood picture" is further altered by characteristic changes in the white blood cell content; the leucocytes elaborated by lymphatic tissues (lymphocytes) increase in number, but those formed in the bone marrow (granulocytes) decrease to such an extent that a lowered total white count results. The entire alteration in the blood picture (both red and white cells) characteristic of pernicious anemia is undoubtedly only a secondary symptom and results from previous changes which have occurred in the bone marrow.

The bone marrow, the tissue in which the red blood cells are formed and in which they develop, undergoes a change in which there is considerable

extension of the red marrow tissue. A large part of the normally inactive yellow, fatty marrow is transformed to the red marrow type of tissue; hence in this disease the bone marrow presents a characteristic appearance of excessive formation of tissue (a hyperplastic condition). In addition there is a striking increase within the tissue of the number of immature, embryonic-like, red blood cells (megaloblasts) and a decrease in the number of mature forms (normocytes and erythrocytes). The diseased marrow itself in many ways appears to have reverted to an embryonic state. During the remission produced by appropriate therapy the marrow regains a more normal appearance because the reverse processes occur; the amount of yellow fatty marrow increases; the immature cells decrease in number; and there is an increase in the number of mature cells which can be spilled into the peripheral blood system.

Malfunctioning of the mucosa of the gastro-intestinal tract is probably the initial cause of the pathological changes occurring in the bone marrow. In true pernicious anemia, this mucosa, particularly the gastric portion, atrophies, markedly reducing the patient's secretion of gastric juice. Achlorhydria (failure to secrete free hydrochloric acid) even following a subcutaneous histamine injection, is a characteristic symptom. Essentially, however, the direct cause of the development of pernicious anemia is probably the inability of the patient to elaborate and secrete a specific substance, a component of gastric juice designated the "intrinsic factor". Extensive histological studies have been made to determine which gastric cells are responsible for the secretion of this intrinsic factor and thus to localize the site of degeneration which leads to pernicious anemia. Following the demonstration by Sharp (1), and Isaacs and Sturgis (2) of the existence of this factor in the stomach lining of swine, several investigations were carried out

using the linings of hog stomachs and intestines to further characterize the intrinsic secretory cell. Meulengracht (3) using swine localized the active substance in the glands at the stomach-small intestine junction (pyloric gland organ), the cells in the body (fundus) of the stomach being active. Activity was also shown to be present throughout the entire small intestinal tract. However, a histological study of stomachs from pernicious anemia patients showed little or no apparent change in the pyloric type cell, but definite degeneration of the fundus type. This was confirmed by Fox and Castle (4). Whatever the nature of the gastric lesion which causes this decreased ability to secrete the intrinsic factor, it does not appear to be the end result of an inflamatory process (5).

Frequently, the conditions just described are accompanied by an involvement of the nervous system, a degeneration of a portion of the spinal cord (subacute combined degeneration). The extent of nervous involvement does not necessarily parallel the severity of the anemia; hence it is not regarded as merely a consequence of the anemia itself but is believed to be directly related to the primary factors responsible for the disease. Degeneration in the peripheral nervous system has also been demonstrated to occur in pernicious anemia. Incoordination and the loss of vibratory sensation, usually in the lower extremities, are the symptoms of nervous system impairment most often observed.

Physiological Changes. The primary physiological disturbances which can be demonstrated in pernicious anemia all result from the decrease in gastric juice secretion. This reduced secretion is directly responsible for the following chemical changes which ensue: (a) the process "catalyzed" by

the "intrinsic factor" of the gastric juice no longer proceeds at a rate adequate for the needs of the patient; (b) an achlorohydria occurs; (c) the liver becomes depleted in a substance(s) designated as the erythrocyte maturation factor. In addition, a number of secondary changes in the patient's metabolism are reflected in alterations which can be demonstrated by chemical tests, but which cannot as yet be logically correlated with the primary disturbances induced by the gastric lesions. The best characterized examples of such changes which occur are: (a) alterations in porphyrin metabolism, (b) a defect in tyrosine metabolism; (c) a decrease in the choline esterase activity of serum and red blood cells; and (d) abnormal concentrations of cholesterol in the blood plasma.

The ability of the gastric mucosa to secrete the essential "intrinsic factor" appears to be irretrievably lost, necessitating the constant treatment of the patient. Reports of the return of normal gastric function following treatment have appeared frequently in the literature; however, the tendency at present is to question the diagnosis of these cases as true pernicious anemia. Likewise, achlorhydria, even following histamine injections, has been found to be a constant feature, both before and after remission in all cases where an adequate and thorough study has been made (6, 7, 8).

It has been demonstrated that the liver stores an erythrocyte maturation factor, necessary for the normal functioning of bone marrow. Depletion of this (9, 10) factor in the liver precedes the development of those pernicious anemia symptoms which are reflected in the hematopoietic picture. Extracts prepared from livers of pernicious anemia patients in relapse, when tested clinically, lacked any anti-anemia activity. If, however, the liver extracts

were obtained from specimens of patients in whom the anemia had been relieved by therapy, the erythrocyte maturation factor was present.

Altered porphyrin metabolism in pernicious anemia is apparent. Bilirubin is present in the blood plasma in significantly increased amounts (11), and the concentration of urobilin and urobilinogen in the feces, duodenum, and urine is increased, a condition usually considered to be indicative of a hemolytic anemia. In support of this concept that hemolysis occurs during pernicious anemia, is the fact that red blood cells of treated and untreated pernicious anemia patients have been transfused into normal individuals and the cell survival time noted. The cells from the untreated patients had a shortened survival time, which may be taken as the criterion for a true hemolytic syndrome (12).

Coproporphyrin I excretion increases during periods of relapse and decreases following remission. Thus, the excretion inversely parallels hematopoiesis, and the amount excreted can be taken as an index (inverse) of the blood regenerating activity. It has been found that whenever hyperplastic bone marrow is present there is a similar increase in coproporphyrin I excretion. The reason for this relationship, however, remains obscure. Two separate lines of porphyrin synthesis (the so-called "dualism" of porphyrin metabolism) exist in man, giving rise to etioporphyrin I and etioporphyrin III. Hematin is derived from etioporphyrin III, but without complete disruption of the porphyrin ring it is impossible to conceive of etioporphyrin III becoming converted to etioporphyrin I. Hence the experimentally observed relationship between the apparent reduced production of hematin and the increased formation of coproporphyrin I is not obvious.

Detection of an increase in blood phenols among pernicious anemia patients has been reported (13). More recently a new study was undertaken in which it

was found that among untreated patients there is an increased excretion of keto acids and hydroxyphenol acids. Following therapy, the amount of these substances excreted returns to normal. The disturbance is apparently in the tyrosine metabolism (14).

Serum and red blood cell cholinesterase activity has been consistently shown to be decreased in pernicious anemia patients in relapse; normal activity values are obtained following the response after the institution of adequate therapy (15, 16, 17).

Similarly the free cholesterol in the plasma of the pernicious anemia patient is reduced but is restored to the normal level during remission (18, 19). Although free cholesterol is diminished in the red blood cells, esterified forms are present in excess of the usual range (20).

Decreased lecithin phosphate in blood plasma frequently occurs; otherwise no other consistent changes in fat metabolism have been noted. The suggestion has been made that this alteration in fat metabolism might be related to the degeneration of the nervous system (21). The question arises whether this decrease is a non-specific secondary metabolic upset, such as one might expect to occur as a result of the primary alteration in the hematopoietic system (e. g., like negative nitrogen balance, altered blood sugar values) or is an effect due directly to a lack of the anti-pernicious anemia factor. Because of the lack of parallelism between the severity of the nervous and hematological involvements one is tempted to postulate a direct, separate influence of the anti-pernicious anemia factor upon each of these systems, and hence possibly a participation of the anti-pernicious anemia factor in phospholipid metabolism.

B.

AGENTS USED IN THE TREATMENT OF PERNICIOUS ANEMIA

Diet had long been considered as a possible factor involved in pernicious anemia, but until 1926 had received no intensive investigation. Noting the favorable results obtained with liver in the experiments of Whipple and Robscheit-Robbins (effect of diet upon blood regeneration following acute hemorrhage)(22), Minot and Murphy in that year treated a series of 45 pernicious anemia patients with a special diet in which liver was a prominent factor (23). The responses which they obtained established the therapeutic value of liver and initiated the attempts to concentrate the active fraction from the whole liver. Some degree of concentration was soon effected, but it was not until 1948 that the isolation from liver of a crystalline compound possessing anti-pernicious anemia activity was announced (24).

These preparations from liver are usually administered parenterally. When given orally a much greater amount of the active material must be given to elicit the same clinical response (25).

In the meantime, Castle had demonstrated that mucosa of the stomach normally secretes a substance (the "intrinsic factor") which acts upon an unidentified dietary material (the "extrinsic factor") to produce an intermediate substance which was believed to be ultimately converted to the erythrocyte maturation factor found in liver (26, 27, 28). Consequently, the therapeutic value of dried stomach preparations was tried and established by the work of Sturgis and Isaacs, and Sharp (1, 2). Thus it is possible to alleviate the deficiency not only by the parenteral injection of the erythrocyte

maturation factor but by supplying orally an exogenous source of "intrinsic factor". Commercial preparations of the "intrinsic factor" vary, some contain the dried stomach mucosa only, others include dried liver concentrates.

In addition to these two substances, a third agent, folic acid, was found to produce definite remissions in pernicious anemia patients. Folic acid is a nutrilite that was discovered and isolated solely on the basis of its growth promoting effects on microorganisms. Later, tested clinically, it was found to stimulate a striking hematopoietic response in pernicious anemia patients (29, 30, 31). The amount of folic required to obtain remission is relatively large in comparison with the amount of liver extract which is effective. No cases responding to liver extracts have been reported in which adequate folic acid therapy could not also produce a hematopoietic response, but it has been observed that folic therapy does not prevent the development of the neurological symptoms. Nor does it arrest the development of those neurological symptoms which may be already present (32).

A number of derivatives chemically related to folic acid have been tested for their hematopoietic activity. Those which have been reported to be active in macrocytic anemia are: (a) pteroyl-α-glutamylglutamic acid (33), (b) pteroyl-γ-glutamyl-γ-glutamylglutamic acid (34), pteroylhexaglutamyl glutamic acid (34), and (d) formylfolic acid (35). None of these compounds show more activity than folic acid.

Thymine, a pyrimidine constituent of thymonucleic acids, can partially replace the folic acid requirement of certain microorganisms. Consequently, it and related compounds were tested clinically. Only thymine, could be demonstrated to have any anti-pernicious anemia activity (32).

In the following discussion, it has been found convenient to divide these therapeutic agents on the basis of both their chemical and functional natures into three groups: (a) the agents directly related chemically to the factor isolated from liver, (designated as erythrotin or vitamin B_{12}), or substances involved in the formation and utilization of this vitamin; (b) compounds derived from folic acid (pterins and p-aminobenzoic acid containing compounds); and (c) nucleic acid components (nucleotides, nucleosides, purines and pyrimidines). In addition to pernicious anemia, there are a number of other disorders which have been found to respond to certain of these agents. Some of these pathological conditions are obviously related physiologically to pernicious anemia by virtue of their also being macrocytic anemias; in others the relationship, if any, is only apparent in that they have therapeutic agents in common with the macrocytic anemias.

C.

PATHOLOGICAL CONDITIONS RELATED TO PERNICIOUS ANEMIA

Although from the standpoint of treatment, pernicious anemia can be classified as a deficiency disease, it is not a deficiency in the usual sense inasmuch as it does not arise from an inadequate dietary regime. True nutritional deficiencies, which result in similar anemias, however, are recognized.

Thus, Wills (36) showed that tropical macrocytic anemia could be treated by supplementing the inadequate diets with marmite, an autolyzed yeast preparation. At first this type of anemia was believed to occur predominantly among pregnant women, but later investigations showed it to be more general in distribution. The alterations observed in the peripheral blood stream and in the bone marrow are characteristic of those present in

pernicious anemia. However, the relative rarity of subacute combined spinal cord degeneration and (the absence of an increased occurrence) of achlorhydria among these patients distinguish the tropical macrocytic anemia from pernicious anemia. Because of the marked similarity to pernicious anemia, liver extract therapy was tried. Satisfactory hematopoietic responses occurred only as long as the less purified preparations were used, indicating that these two types of macrocytic anemias differed in their response to therapeutic treatment (37, 38).

A macrocytic anemia sometimes occurs during pregnancy. As in pernicious anemia, a megaloblastic, hyperplastic bone marrow is constantly encountered, and though subject to some variability, the peripheral blood picture is usually similar to that found in pernicious anemia. At first considered to be of nutritional origin, this condition was later shown to be present among individuals eating apparently adequate diets. However, as in tropical macrocytic anemia, cases have been reported which were refractory to purified liver extracts (rich in vitamin B_{12}) but which responded to the administration of folic acid (39). Termination of pregnancy is followed by remission of the anemia without the continuance of therapy. That the anemia might be the result of an increased need for folic acid occasioned by the fetal demands upon the mother may be postulated and this could also account for the long lag between the institution of therapy and the beginning of remission. Certain discrepancies, however, remain to be answered. Thus, why will orally administered liver extract sometimes succeed in relieving the anemia where parenteral administration failed(40)? Also what evaluation should be given to the evidence that in some cases secretion of the "intrinsic factor"

apparently ceases but returns following parturition (41)?

Correlating evidence for the inadequacy of the erythrocyte maturation factor to function in replacing folic deficiencies has been obtained through the use of experimental animals. Reports have been made concerning the inability of folic-free liver extracts (rich in vitamin B_{12}) to replace the need for folic acid in nutritional deficiencies (42, 43)in the deficiency induced in rats by succinylsulphathiazole (44), and in the deficiency induced by X-methylfolic acid in dogs (45).

Sprue, ideopathic steatorrhea, and celiac disease present similar clinical pictures usually showing certain symptoms common to the macrocytic anemias. In these disorders, however, there is a marked intolerance for fats, and often for carbohydrates, and diarrhea is a predominant symptom in most cases. A characteristic distortion of the intestinal mucosa is usually revealed by roentgenographic examination of the intestine. This pathological development results in a decrease in the absorptive capacities of the intestinal mucosa, a condition which can be demonstrated by a glucose tolerance test (46). Decreased absorption of xylose can also be demonstrated (47).

Sprue is usually classed as a nutritional deficiency. However, it seems to occur among both the well-to-do and the indigent; in fact, Ashford reports the disease to be more prominent among the former (48). The nutritional deficiency undoubtedly arises as a result of the decreased intestinal absorption. But it cannot definitely be stated that this intestinal change is the result of rather than the cause of the deficiency.

The peripheral blood picture in sprue may be either that of a hypochromic, microcytic anemia (as in iron deficiency)(49) or of a macrocytic anemia (50). The latter is more common and is identical with that found in pernicious anemia. Cases have been observed where microcytic anemias have given way to macrocytic anemia. Leukopenia frequently accompanies the macrocytic anemia.

Bone marrow from cases with microcytic anemia typically contains no megaloblasts while marrow from patients with macrocytic anemia has the megaloblastic appearance encountered in pernicious anemia. Generally sprue cases are not seen until well advanced. It is possible that the incidence of macrocytic anemia in sprue would be less if the cases were detected in the early stages of the disease.

Probably the macrocytic anemia of both sprue and pernicious anemia results from a lack of the erythrocyte maturation factor. In the latter dyscrasia this lack is attributed to the failure of the individual to secrete the "intrinsic factor" necessary for the utilization of the dietary "extrinsic factor" which is elaborated into the erythrocyte maturation factor. The deficiency in sprue, however, arises not from inability to produce the erythrocyte maturation factor, but from defective absorption.

Achlorhydria is not a constant feature of sprue, and even among those cases in which the absence of free hydrochloric acid secretion has been reported are some in which secretion has returned following therapy (51).

Unlike pernicious anemia, adequate production of "intrinsic factor" takes place in all cases of sprue. Those cases in which its presence cannot be

demonstrated should be classified as pernicious anemia. This indicates the difficulty sometimes encountered in differentiating one dyscrasia from the other.

Sprue appears to be a disease entity in which either folic acid or liver extract (containing vitamin B_{12}) can be used with satisfaction. After remission adequate dietary therapy should still be maintained, since a low intake of fat is necessary to prevent the steatorrhea (52, 53). Once the anemia has been relieved, the intestinal absorption usually improves and the administration of the hematopoietic substance can be discontinued. Relapses, however, are likely to occur unless there is strict adherence to the diet restricting fats and carbohydrates.

It is interesting to note that only rarely have cases of sprue been reported to have the spinal cord degeneration found in pernicious anemia (54). This fact coupled with the effectiveness of either folic acid or liver extract therapy would appear to place sprue intermediate between tropical macrocytic anemia and pernicious anemia from the standpoint of the chemical factors involved in precipitating the pathological conditions.

The nutritional deficiency of an "animal protein factor", now shown to be identical with the vitamin B_{12} (55, 56), has been noted to occur in chicks (57), rats (58), and mice (59). Anemia, leucopenia, decreased growth rate and death result from a lack of this factor, and it has been demonstrated that folic acid cannot be substituted for this factor in the treatment of these deficiencies (57). Death in the weanling rat appears to be due to hemorrhage in the fundic portion of the stomach.

There has been no clear cut demonstration of a nutritional deficiency

of the "extrinsic factor". Inadequate intake of this factor has been postulated as a contributing factor in those cases of pellagra exhibiting a macrocytic anemia. Usually, however, the deficiency in most pellagrans is of such a multiple nature that the clinical pictures have few constant features apart from certain characteristic skin and mucous membrane lesions. In some pellagrans a diminished secretion of the "intrinsic factor" has been reported (60).

While macrocytic anemia is not a prominent feature of fish tapeworm (Diphyllobothrium latum) infestation, when this symptom is present, it gives a clinical picture very similar to that of pernicious anemia. Spinal cord involvement is rarely encountered. Response of this type of anemia to liver extract therapy is similar to that for pernicious anemia.

Certain anomalies have been observed concerning the development of a macrocytic anemia following infestation by the fish tapeworm. The incidence of macrocytic anemia is not always proportional to the extent of the infesting worm population. Areas where pernicious anemia is more common have a higher rate of the macrocytic anemia associated with worm infested persons. Familial tendencies have also been noted. This associated anemia might thus be but a matter of coincidence, where infestation and pernicious anemia have occurred simultaneously; or possibly there exists a constitutional predisposition to pernicious anemia among those in which the condition develops, which would not have been detected had they not been infested by the tapeworm (61).

If the parasite is responsible, the problem of how the anemia is produced must be answered. Interference with gastric secretion, interference with absorption, or removal of the erythrocyte maturation factor have all been suggested as possible modes of action. Recently the location of the infestation

has been reported as relevant to the development of anemia, macrocytic anemia resulting only when the parasites inhabit the small intestine (62).

In stomach carcinoma one might expect interference with the normal secretory functions of the stomach. Thus a lack of "intrinsic factor" can be postulated as the basis for the relatively few cases of macrocytic anemia observed to occur as a consequence of stomach carcinoma, and relieved by parental injection of liver extract (63, 64, 65). There is evidence of a significantly higher incidence rate of stomach carcinoma among persons treated for pernicious anemia than there is among persons free of this dyscrasia (66, 67).

In man the secretion of "intrinsic factor" occurs mainly in the fundic portion of the stomach. For pernicious anemia to result from gastrectomy, it would have to be total and include the entire cardiac region. It is the opinion of Bethell, et al., that following total gastrectomy pernicious anemia will eventually develop (68).

Cases of partial gastrectomy and gastro-enterostomy have been reported to result in a macrocytic anemia. The number, however, when compared to the total number of such operations performed is relatively small.

Among the cases of fully developed intestinal strictures or anastomoses are many which present a clinical picture typically that of pernicious anemia. The peripheral blood pictures are identical; a hyperplastic, megaloblastic bone marrow is present; leucopenia is common; however, the neurological involvements are not pronounced. As might be anticipated, achlorhydria is not a prominent feature, nor is there a decrease in the excretion of "intrinsic factor" (69). Cases have been reported in which operation for correction of

the intestinal condition has resulted in relief of the anemia (70). Deficient absorption would seem the logical explanation; however, studies have shown glucose and fat absorption to be relatively good. On the other hand, the liver extracts which are used to relieve the anemia must be given parenterally since oral administration is ineffective.

The extent to which macrocytic anemia occurs in association with liver disease remains in doubt. In rats with a carbon tetrachloride induced cirrhosis of the liver, a progressive development of macrocytic anemia has been noted as the cirrhosis increases (71). Reports on macrocytic anemia accompanying liver disease in man show a variation from an 0 to 91 percent incidence (72, 73). The anemia never becomes as severe as it does in pernicious anemia. Clinically the symptoms are generally those associated with the liver disease rather than the anemia. Achlorhydria was found in only 40 percent of the cases with macrocytic anemia (74). On the basis of the reported ability of liver to store the erythrocyte maturation factor, the suggestion was made that inability to store this factor is the precipitating factor in development of a macrocytic anemia. However, extracts prepared from diseased livers of patients in whom there had been an accompanying macrocytic anemia showed anti-pernicious anemia activity when tested clinically indicating no impairment of storage ability (75). Alternatively, it is proposed that the erythrocyte maturation factor must be acted upon in some manner by the liver. This would account for failure of the anemia to respond satisfactorily to liver extract therapy (76, 77). Cases have, however, been reported in which the anemia does respond to liver extract (78, 74).

In addition to these macrocytic anemias, another, designated achrestic anemia, has been described in which the morbid anatomy is similar to that of pernicious anemia, but which is unrelieved by either liver extract or folic acid therapy (79). Unlike pernicious anemia, subacute combined degeneration of the spinal cord does not occur. Also free hydrochloric acid can be demonstrated in the gastric juice. Liver extracts prepared from several of these cases were demonstrated to contain the erythrocyte maturation factor. Apparently this is a condition in which the bone marrow becomes megaloblastic not from a lack of the erythrocyte maturation factor but from an inability to use it.

D.

THE CHEMICAL NATURE OF THE THERAPEUTIC AGENTS USED IN THE TREATMENT OF MACROCYTIC ANEMIAS

Many attempts have been made to clarify the character of the intrinsic and extrinsic factors and the nature of their reaction. A prime difficulty in their characterization has been lack of an adequate, accessible means of assaying for activity.

Intrinsic factor. In general an enzymatic character is proposed for the intrinsic factor. Among its protein-like attributes, the following can be listed: (a) thermolability (destroyed by boiling neutral solution for 5 minutes, by holding at a temperature of 70-80°C. for one half hour or at 40°C. for three days), (80) (b) destruction by autolysis of gastric juice, (c) destruction by prolonged digestion with pepsin or trypsin (81), (d) precipitation by saturation with ammonium sulfate, (e) non-dialyzable, (f) non-ultrafilterable, (g) about 50 percent adsorption at pH 7.4 by an amount of

Lloyds reagent required for complete removal of pepsin and rennin from the gastric juice (82, 83). More recently a report has been made that contrary to the above work the intrinsic factor is ultrafilterable (84).

Studies have been made on the proposal that the intrinsic factor possesses proteolytic activity (85, 86, 87, 88), but no definite information has been obtained. Agren believes the intrinsic factor to be identical with aminopolypeptidase on the basis that they appear to be concentrated in parallel (89). Anderson and Faber, however, were unable to confirm Agren's work (90).

Extrinsic factor. In contrast to the intrinsic factor, the extrinsic factor appears to be a highly stable substance. Thus it resists autoclaving for five hours at 15 lbs. pressure (91), hydrolysis with dilute sulfuric acid (91), and treatment with alkali (92). Dilute acetic acid has been used to prepare extracts from muscle which contain extrinsic factor activity (92). It is also soluble in 80 percent alcohol (91).

Several materials have been tested for their content of extrinsic activity. Due to the limitations imposed by the assay procedure, extensive surveys have been impossible. Many of the negative results reported prior to 1936 must be discarded following the experiments of Castle and Ham (93) in which they showed that prolonged incubation at too high hydrogen ion concentrations resulted in a loss of activity.

Since one or more members of the B vitamin complex might constitute the extrinsic factor, Castle, et al. (92), tested the following combination of substances: thiamin, riboflavin, nicotinamide, pyridoxine hydrochloride, calcium pantothenate, para-aminobenzoic acid, choline hydrochloride, inositol,

biotin, xanthopterin and folic acid. No indication of extrinsic activity was discernable.

Egg white (94), Cohn's liver fraction "G" (95), autolyzed yeast, rice polishings, wheat germ (96), and crude casein (97), in addition to beef muscle have been found to contain the extrinsic factor. Yeast has been a particularly controversial source. Wills (98) proposed that autolysis of yeast was necessary to release the extrinsic factor. The question concerning autolysis is complicated since there also is a question as to whether or not yeast in the absence of the intrinsic factor has hematopoietic activity. The number of contradictory results led Wintrobe to conduct a more comprehensive investigation of the situation. In trials with fifteen cases, he found dried brewer's yeast yielded a maximum response in five cases. A sub-maximum response was obtained in only two cases, both of which were also later found to respond inadequately to liver therapy.(Baker's yeast and extracts therefrom proved to be unsatisfactory). Amounts of beef, equivalent on a protein basis to the brewer's yeast, when fed to five patients elicited no response in four, the fifth individual responded but also gave an additional secondary response when fed yeast. Incubation of dried brewers' yeast with gastric juice effected an increase in activity comparable to that obtained when liver was similarly incubated (99). In view of the responses subsequently obtained with folic acid, it becomes apparent that part of the observed activities could be attributed to this compound.

Reaction between the intrinsic and extrinsic factors. The nature of the reaction between the postulated extrinsic and intrinsic factors has thus far not been found. From Castle's experiments (93) it is apparent that

contact between the two factors is necessary. Thus if they are fed separately and an interval of longer than six hours is allowed to lapse between their administration, no activity can be demonstrated.

The reaction itself appears to occur normally in the gastro-intestinal tract. It was found that a pH from 5-7 furnished consistently better results. A low pH or a prolonged incubation of mixtures containing the two substances yielded inactive material (93).

Greenspon (100) suggested that the pepsin in an acid medium inactivated the intrinsic factor and that the role of the extrinsic factor during incubation was to remove or inactivate the pepsin, thus protecting the intrinsic factor and leaving it potent and intact. However, after the intrinsic factor and pepsin have been separated, the former continues to be inactive unless it can contact the extrinsic factor.

The production of anemia by fish tapeworm infestations is related to the location of the parasite in the intestine. When there is appreciable infestation of the small intestine a macrocytic anemia is usually present. In such cases, where there is no deficiency of either the extrinsic factor in the diet or of the intrinsic factor in the gastric secretion, there must be either (a) a further transformation involving an intestinal factor which can no longer take place due to the infestation; or (b) a removal or destruction by the parasite in the intestinal tract of one of the components or intermediates of the system involved in producing the erythrocyte maturation factor.

Efforts have been made to obtain the erythrocyte maturation factor from incubation mixtures of extrinsic and intrinsic preparations. The results do not appear to be decisive in demonstrating either the success or failure of these attempts. On the basis of Castle's work it would appear that

in vitro incubation does not yield the erythrocyte maturation factor; and he has postulated the formation of a thermolabile intermediate (101).

Klein and Wilkinson (102), however, reported the preparation of a thermostable extract from an incubation mixture using the same methods that have been employed for the preparation of potent liver extracts. Their intrinsic source was the press juice of hog stomach which they had fractionated. After six hours of incubation at 37°C., a clear liquid was separated from the mixture (150 g. fresh beef, 5 g. stomach fraction) and heated to 60-65°C. for a period of thirty minutes. When tested by oral administration, this liquid preparation was found to be active. The *unheated* stomach fraction was active by itself, but after comparable heating no response was obtained when it was administered either alone or following a six hour incubation with fresh beef. These results point to the formation of a distinct reaction product.

Supplementary evidence along the same line was supplied by Kyer, Brooks, and Isaacs (103). Using desiccated stomachs they prepared acid and aqueous extracts. Heating these extracts to 60-70°C. for one-half hour inactivated them. Yet, if the acid extract were allowed to incubate eight hours at 37°C. before such heatings, the preparation remained active, although it was possible to inactivate the active principle by raising the temperature to the boiling point.

Attempts to prepare an active erythrocyte maturation factor extract from dried swine stomachs using the concentration methods employed in manufacturing liver extract preparations have been reported as unsuccessful (104).

Mazza has reported (105) the preparation of a thermostable erythrocyte maturation factor by incubation. In this case also (as in Wilkinson's report)

press juice of macerated hog gastric mucosa membrane was used as the source of the intrinsic factor. Ox muscle was used to supply the extrinsic factor. Following incubation at 37°C., the formation of a thermostable anti-pernicious anemia factor was claimed.

Rossi (106) incubated a filtrate fraction obtained from yeast with human gastric juice. Tested separately neither the yeast extract nor the gastric juice showed hemopoietic activity, but by incubating them together (6 hours at 37°C.), activity was developed. The active principle was heat stable, soluble in 70 percent alcohol and insoluble in 90 percent alcohol.

Working with dried stomach preparations presents several disadvantages. In general such preparations contain a sufficient amount of extrinsic factor to show hematopoietic activity alone. Nor can one rule out the possibility that these preparations might contain intermediary reaction products. The intermediary reactions which take place between the initial incubation of extrinsic and intrinsic factors and the final formation of the erythrocyte maturation factor as it is isolated from liver remain unknown. The evidence cited gives some basis for believing that the initial reaction between the intrinsic and the extrinsic factors occurs enterally and that an additional intestinal factor may be involved.

Jacobson (107) reported finding the absence or marked diminution of argentaffine cells from the gastro-intestinal tract of pernicious anemia patients. Wilkinson (108) separated the layers of hog duodenum and tested them clinically. He found(in accordance with Jacobson's postulation) that the exterior fraction consisting of the villi (including argentaffine cells) and crypts of Lieberkuhn (99 percent) and Brunner's glands (1 percent) gave a hematopoietic response. The intermediate layer (66 percent Brunner's glands

and 1-2 percent villi and crypts), and duodenum muscle layer were both without hematopoietic activity. It is possible that these argentaffine cells serve as the site for (a) the formation of either the thermolabile intrinsic factor itself, or for (b) the production of a relatively thermostable intermediate in the formation of the erythrocyte maturation factor, or for (c) the final reaction in which is formed the erythrocyte maturation factor itself.

While the site of secretion and the site of reaction do not necessarily coincide, it is hardly probable that the reaction site would be located above the secretion site.

Erythrocyte maturation factor. The erythrocyte maturation factor is very heat stable and will withstand autoclaving for five hours at 15 lbs. It is both acid and alkali labile.

Concerted efforts were made to isolate the active principle from liver preparations. From the study of Cohn and collaborators (109)it was suggested that the active component was a nitrogenous base having a low nitrogen content (conditions excluding such possibilities as purines and pyrimidines). Dakin, Ungley, and West (110) postulated that the erythrocyte maturation factor was a peptide or at least associated with a peptide. Karrer, Frei, and Fritzsche (111) reported an active preparation which contained no phosphorous, pentose, flavin, pterin or reducing carbohydrate. SubbaRow, Jacobson, and Fiske (112, 113) obtained results indicative that actually a combination of two or more substances yielded more successful therapeutic results. On the basis of this experimental work, a multiple factor hypothesis was evolved. Later trials indicated that there was a primary hematopoietic factor in liver and in addition at least three accessory factors (114). A complex purine, a peptide and l-tyrosine have been postulated as composing these accessory factors.

Other groups have also postulated the probability of more than one factor. Eisler, Hammersten, and Theorell (115) separated liver extract into two portions, one of which initiated the production of young, reticulated red blood cells (reticulocytosis) and the other, when administered with the first, caused their further development into normal red blood cells. The fraction stimulating reticulocyte formation could be replaced by definite chemical compounds, as, for example, the protein-free salt of thymonucleic acid.

Wichels and Hofer (116) similarly separated a reticulocyte factor from preparations possessing a general curative activity.

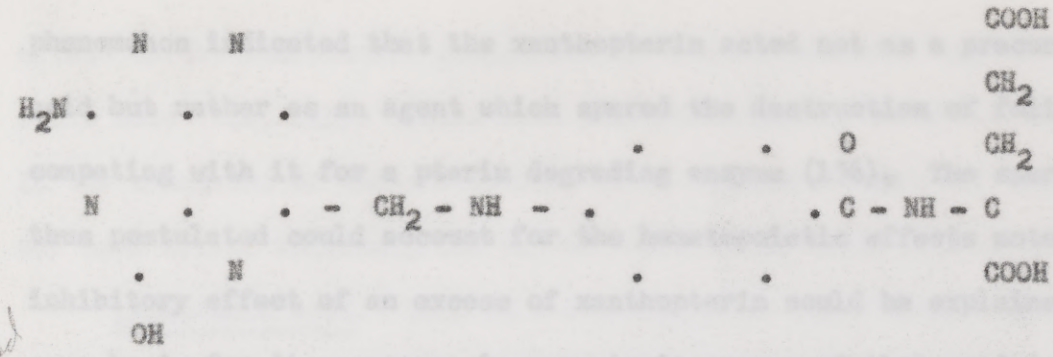
A crystalline erythrocyte maturation factor was finally isolated in the spring of 1948 (24, 24a). This material was active clinically in µgm amounts. It was described as small, red, needles which darkened at 210-220°C., but did not liquify below 300°C. Emission spectrographic analysis showed the presence of cobalt. No sulfur, but phosphorous and nitrogen are present in this cobalt complex. The absorption spectrum corresponds to those of a porphyrin compound. Using micro-biological assays, it was shown that this compound is destroyed by standing at room temperature for 23 hours in dilute acid (0.001 N) or alkali (0.015 N)(117).

Folic acid. Wills' observation in 1932 (36) that marmite could be effectively used to relieve tropical macrocytic anemia was probably the first concerned with a biological effect that could be attributed to what we now know is folic acid. It was found that an analogous deficiency could be obtained experimentally by subjecting monkeys to a similar environment (118, 119). Cytopenia, a reduction in the number of circulating blood cells, was the predominant feature, and was the criterion which gave the most accurate

estimate in animal assay procedures. The factor necessary to alleviate the deficiency was termed vitamin M (120, 121).

In 1940, Snell and Peterson (122) reported that an unidentified factor was necessary for the growth of Lactobacillus casei, and they termed it the "norite eluate factor". The same year the importance of vitamin B_c, as a dietary factor which prevented chick anemia was also announced (123). The following year it was found that concentrates of the "norite eluate factor" were also active in promoting the growth of Streptococcus faecalis R (formerly known as Streptococcus lactis R)(124). A highly refined preparation of a factor prepared from spinach by Mitchell, Snell and Williams and designated as folic acid was shown to be the active principle promoting the growth of Lactobacillus casei and Streptococcus faecalis R in a medium which was otherwise well characterized (125).

A crystalline preparation of the chick anti-anemia factor (vitamin B_c) was isolated in 1943, and its activity as a growth factor for both Streptococcus faecalis R and Lactobacillus casei was demonstrated. Hence it was postulated and subsequently demonstrated that the micro-biological activity of the norite eluate factor and folic acid were identical with each other and with vitamin B_c (126). The structure of this vitamin was shown to be N-pteroyl-L-glutamic acid in publications which simultaneously announced the proof of its structure and its synthesis (127, 128).



N-pteroyl-L-glutamic acid

Broken down into its component parts, the folic acid molecule is composed of (a) an aminohydroxypterin (related to xanthopterin) connected by a methylene bridge to (b) p-aminobenzoic acid which is linked by a peptide bond to (c) glutamic acid. Significant physiological activities had previously been ascribed to each of these three component parts.

Xanthopterin. The hematopoietic effects of this compound have been observed on a number of occasions. It effectively relieves the anemia of baby rats nursed on goats' milk (129), and an anemia in young Chinook salmon (130, 131). Alleviation of vitamin M deficiency (i. e. folic acid deficiency in the monkey) by xanthopterin has also been reported. The effect here, however, is only temporary and is also incomplete (132). Reports that xanthopterin stimulates the rate of cell proliferation (both red blood cells and nucleated cells) in bone marrow suspensions have been made. Similar results have been noted in nutritionally anemic rats. The amount of pterin used seems to be critical; above a certain concentration the compound loses its stimulatory capacity and becomes inhibitory (133).

At one time it was thought that xanthopterin could be used by liver homogenates to synthesize folic acid (134, 135). Further investigation of this

phenomenon indicated that the xanthopterin acted not as a precursor of folic acid but rather as an agent which spared the destruction of folic acid by competing with it for a pterin degrading enzyme (136). The sparing action thus postulated could account for the hematopoietic effects noted. The inhibitory effect of an excess of xanthopterin could be explained upon the same basis for its presence in amounts in excess of that needed to produce the sparing effect would undoubtedly cause it to compete with folic acid for a place in the enzyme systems in which folic acid functions.

Xanthopterin has also been reported to inhibit the development of tumors (137, 138), an observation which may be related to the inhibition of Rous Chicken Sarcoma by a nutritional deficiency of folic acid (139,140).

p-Aminobenzoic Acid. The biological role of this compound was first recognized through its ability to prevent the sulfonamide inhibition of the growth of micro-organisms. It was postulated that the sulfa drugs exerted their activity by competing with p-aminobenzoic acid for an enzyme system (141, 142). Subsequently it was shown that yeast extracts could be used to reverse the sulfonamide inhibition. The isolation of p-aminobenzoic acid from these yeast extracts established its natural occurrence (143).

After the elucidation of the structural composition of folic acid, it was postulated that biochemically p-aminobenzoic acid functioned primarily in the synthesis of the former compound. This would explain why a nutritional folic acid deficiency (which cannot normally be produced in rats due to bacterial synthesis of this vitamin in the rat's caecum) can be induced by the oral administration of sulfonamides. These drugs inhibit the bacterial synthesis of folic acid from p-aminobenzoic acid to the extent that the usual physiological manifestations of a folic acid deficiency appear. Usually the

effect of the sulfonamide antagonism of enteric bacteria can be reversed by folic acid (144, 145). Folic acid has been reported to be clinically active in the treatment of certain anemias of an aplastic character some of which may have arisen following the use of sulfa drugs (146, 147, 148).

L-Glutamic acid. This is a non-essential amino acid. Glycogenic, it yields α-ketoglutaric acid on deamination. Substitution of D-glutamic acid for the L-form in folic acid yields an inactive compound (149).

Derivatives of folic acid. A crystalline conjugate of folic acid, pteroylhexaglutamylglutamic acid, was isolated from yeast (150). This compound was active for the chick, but only 2 to 5 percent as active micro-biologically as folic acid. It has since been shown that this is heptaglutamate, the form of the vitamin predominantly present in the vegetable kingdom (151). In the animal, free folic acid is apparently released from the conjugate through a specific enzyme action (152).

Pteroyltriglutamic acid ("fermentation Lactobacillus casei factor") was isolated in crystalline form (153), and shown to be active for Lactobacillus casei and animals but only slightly active for Streptococcus faecalis R.

Another naturally occurring member of this family which has been isolated is N^{10}-formylpteroic acid (rhizopterin)(154), which is active for Streptococcus faecalis R, but inactive for Lactobacillus casei and animals (155, 156, 35).

Though folic acid is the most widely and efficiently used member of this family of compounds, there is a general feeling that it does not represent the biologically active form. Because of this, numerous attempts have been made to alter it in an effort to obtain a more active compound. The presence of the conjugase enzyme in animal tissues and the isolation of the naturally occurring

tri- and heptaglutamates have led to the investigation of various synthetically produced compounds in which the number and linkage of glutamic acid molecules have been varied.

Such synthetically produced members include: (a) pteroic acid, active for Streptococcus faecalis R, inactive for animals and Lactobacillus casei (157), (b) pteroyl-γ-glutamylglutamic acid, active for Lactobacillus casei and Streptococcus faecalis R (158), (c) pteroyl-α-glutamylglutamic acid, inactive for Lactobacillus casei and Streptococcus faecalis R, but reported active for man (159, 160), and (d) formylfolic acid, active in man (35) and microbiologically (161).

Purines, pyrimidines, and their derivatives. In addition, certain compounds, related functionally rather than chemically to the erythrocyte maturation factor and folic acid, must be considered. Thymine was demonstrated by Spies (162) to relieve the anemia in pernicious anemia, although there was a continued progression of the glossitis and stomatitis symptoms which usually accompany the reduced blood count.

Other purines and pyrimidines have been tested and found to be clinically inactive. The effect of nucleic acids remains controversial; hematopoietic response has been attributed to a mixture of nucleic acids (163), but Strauss and Castle reported nucleic acids to be inactive even after incubating with gastric juice (91). A mixture of nucleotides is usually administered in cases of agranulocytosis, a deficiency of a certain type of white blood cells. There is some evidence that the adenine content is responsible for the therapeutic effect (164).

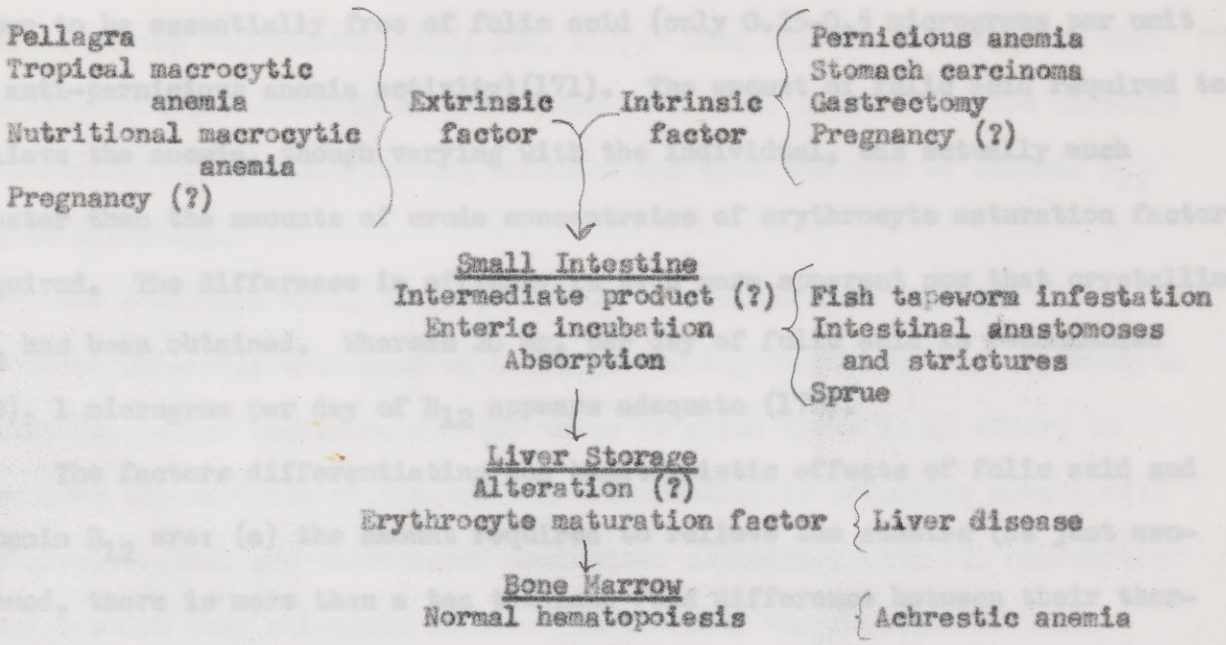
The functional relationship between folic acid and purines and pyrimidines

was first shown in micro-organisms. The folic acid requirements of three species of Lactobacilli were found to be adequately taken care of by adenine and thymine; other purines, guanine and xanthine could replace the adenine (but were not as active); however other pyrimidines could not be substituted for thymine (165). When Lactobacillus casei is inhibited by methylfolic acid it is found that the presence of adenine, guanine, hypoxanthine or xanthine reduces to one third the amount of folic acid needed to overcome a given amount of its antagonist. Alone, thymine had no effect, but in the presence of the purines, thymine reduced the critical amount of the acid still further - to one tenth that required when both purines and thymine were omitted from the medium (166). Thymine has likewise been found to substitute for p-aminobenzoic acid (167).

The desoxyribosides of purines and pyrimidines can be substituted for the vitamin B_{12} requirements of several micro-organisms (168, 169, 170).

Summation and Hypotheses

Generalized scheme to indicate possible relationships between the recognized macrocytic anemias. At the time our investigation was initiated in 1947 a reasonable hypothesis could have been formulated in which the ultimate cause of all the macrocytic anemias could have been attributed to a common cause, namely a specific erythrocyte maturation factor. This difficulty could evolve through a number of pathways and account for the variety of etiological origins of macrocytic anemias. Thus one could visualize a general pattern with interfering factors which might be represented as follows:



In view of the above scheme, it would be reasonable to expect the erythrocyte maturation factor to relieve all the macrocytic anemias with the exception of achrestic anemia. That the explanation, however, is not so simple became apparent as the liver extracts used therapeutically became more and more refined. Later, the clinical trial of folic acid, demonstrating its marked hematopoietic effect among the macrocytic anemias, opened new speculation (29, 30, 31).

The inadequacy of the scheme just presented can be accounted for in one of two ways. Either (a) there is no single basis for the evolution of the macrocytic anemias, or (b) an interrelationship exists between these anemias which at the present is not apparent. As to which alternative applies cannot be established yet due to our limited knowledge of how and where the various substances which are therapeutically active exert their effect.

Non-identity of folic acid and the erythrocyte maturation factor. That folic acid was not the erythrocyte maturation factor of liver was realized even before clinical trials were made, for highly active liver fractions could be

shown to be essentially free of folic acid (only 0.25-0.5 micrograms per unit of anti-pernicious anemia activity)(171). The amount of folic acid required to relieve the anemia, though varying with the individual, was actually much greater than the amounts of crude concentrates of erythrocyte maturation factor required. The difference in efficacy is even more apparent now that crystalline B_{12} has been obtained. Whereas 20 mg. per day of folic acid is recommended (30), 1 microgram per day of B_{12} appears adequate (172).

The factors differentiating the hematopoietic effects of folic acid and vitamin B_{12} are: (a) the amount required to relieve the anemias (as just mentioned, there is more than a ten thousand fold difference between their therapeutic doses); (b) the much more universal hematopoietic effect exerted by folic acid than by the erythrocyte maturation factor (thus, no cases have been demonstrated of folic acid as being ineffective where liver extract would relieve the anemia, but conversely, macrocytic anemias are now recognized which respond to folic acid but not to folic acid-free liver extracts)(173, 174, 175).

In the combined system syndrome of pernicious anemia, folic acid effectively relieves the hematological symptoms, but fails to halt either the development or progression of neurological symptoms. Some individuals even claim that the administration of folic acid may accelerate the development of neurological symptoms (176).

The inability of B_{12} to substitute for folic acid in nutritional or inhibitor induced deficiencies has been mentioned (p.). Folic acid deficiency seems to strike primarily at the myeloid (bone marrow) tissue, resulting in an absolute cytopenia. This relation has formed the basis for the use of inhibitory folic acid analogues in myeloid leukemias. Similarly, folic acid is used therapeutically to combat the leucopenia due to irradiation or other

myeloid tissue depressants (177, 146, 147). Such information suggests that the hematopoietic activity of folic acid in myeloid tissues is more directly concerned with the blood forming function and that the role of vitamin B_{12} may be secondary.

The available data, in general, can be divided into two main categories: (a) those primarily concerned with the extrinsic, intrinsic, and erythrocyte maturation factor sequence, and (b) those in which there is an effort to correlate the activity of the various therapeutically effective agents with the physiological and biochemical deviations associated with the macrocytic anemias which they alleviate in order to indicate the function of these factors in cell metabolism.

Relations of various substances active therapeutically to the extrinsic, intrinsic, and erythrocyte maturation sequence. Non-identity of folic acid and the extrinsic factor. The possibility that folic acid might be the dietary "extrinsic factor" was early postulated. A mass action effect could be proposed to account for its activity in pernicious anemia (and the high level required) since Goldhamer (178) had demonstrated the presence, although in a greatly diminished amount, of intrinsic factor among pernicious anemia patients. However, incubation with gastric juice failed to increase the hematopoietic activity of folic acid (179). Later folic acid was found to be ineffective in the prevention of the neurological symptoms resulting from the combined system deficiency of pernicious anemia (176, 180). Folic acid was thus absolved of the role of extrinsic factor.

Direct and indirect implications of the intrinsic factor in the utilization of folic conjugates. After the isolation of folic acid conjugates,

there appeared reports concerning the inability of pernicious anemia patients to use these compounds (i. e., no therapeutic response comparable to the one ellicited by folic acid)(181, 182). Nor was there an increase in the amount of free folic acid excreted, whereas normal individuals given folic conjugates responded with an increase in the amount of free folic acid excreted. About the same time, the presence of a conjugase (an enzyme which releases free folic acid from its glutamic conjugates) inhibitor was reported to be present in natural materials containing conjugates (183). The inability of the anemic patients to utilize folic acid conjugates could logically be due to a lack of this conjugase, or to an inability to cope with a conjugase inhibitor.

The type of the reaction catalyzed by the conjugase enzyme shows it to be of a proteolytic character. Since the literature contains several reports postulating a proteolytic activity for the intrinsic factor, the trial of gastric juice as a conjugase was logical. No indication of conjugase activity, however, was obtained.

The intrinsic factor could also be implicated as participating in another way in the utilization of the conjugate. Thus the possibility existed that its role was not a direct action upon the extrinsic factor, but rather an inactivation of a conjugase inhibitor, allowing normal digestion and utilization of the conjugate. Consideration of Jacobson's work seemed to fit in with such a scheme. He demonstrated that argentaffine cells contain granules, apparently composed of xanthopterin. Since these cells were shown to atrophy in pernicious anemia, they were believed to be a source of the intrinsic factor (107). The folic acid content of liver homogenates was increased upon incubation with xanthopterin (171, 135). From these observations

it could be postulated that the intrinsic factor is xanthopterin or a derivative thereof secreted by argentaffine cells and that the substance excreted functions by countering the effect of a conjugase inhibitor present in natural substances.

Now, however, all the recent evidence indicates that there is utilization of the conjugates by pernicious anemia patients (184). It has also been demonstrated that although the conjugase inhibitor is active in vivo, its effect in the normal and the anemic patient is the same (185).

Bruyze and Engel (186, 187) claim to have detected a change in the nature of the folic acid conjugates following incubation with gastric juice at pH 2.0. The exact nature of the change is unknown; it is detected by the failure of conjugase preparations to release free folic acid when it is allowed to act upon the altered compound. Since it is known that (a) the inhibitory effect which glutamic acid has upon conjugase activity, and (b) that pepsin, present in gastric juice, is active at pH 2.0, but inactivated by a pH of 7.0, one might postulate that the alteration may be only the freeing of 1 or 2 molecules of glutamic acid. The compound so formed would not of itself be refractory to conjugase, rather there would be an inhibition of the conjugase system by the freed glutamic acid.

The relation between the erythrocyte maturation factor and the metabolism of folic acid. The influence of vitamin B_{12} upon folic acid metabolism or the utilization of stored folic acid remains problematical. It has been reported that initiation of liver extract therapy in sprue causes a large increase in the amount of free folic acid excreted, though no similar occurrence has been noted in pernicious anemia (188, 189).

It has also been reported that liver extract appears to increase the ability of pernicious anemia patients to form free folic acid from its conjugates. This beneficial effect upon folic acid liberation appeared even when the liver extract was tested in vitro with bone marrow suspensions (190). Recently B_{12} specifically has been implicated in the formation of the conjugase enzyme (191). Thus chick livers from day old progeny of B_{12} depleted hens show very slight conjugase activity. If, however, the liver homogenate is incubated with B_{12} an increased conjugase activity develops.

Enzymatic conversion of folic acid to a form with increased hematopoietic activity. There has been much conjecture as to the possibility of enzymatic activation of folic acid. Jacobson and Good (192) treated folic acid samples with a xanthine oxidase preparation (from liver), and claimed on the basis of clinical trials to have obtained a product with greatly increased activity. No further reports of such an activation appeared until Norris published the results of a series of experiments concerning the hematopoietic activity (in vitro) of a substance, designated vitamin B_{14}, isolated from urine (193). The chemical nature of this substance was not indicated, but in the same series of publications xanthopterin and folic acid, following incubation with xanthine oxidase, were reported to exert a similar hematopoietic effect. The inference is that vitamin B_{14} is a pterin compound, possibly folic acid, which has been activated by the oxidase enzyme. Xanthine oxidase activity was also reported to exist in the mucosa of rat stomachs. On this basis it was postulated that there is no one substance existing as the extrinsic factor, but rather a series of pterin compounds. In this scheme, xanthine oxidase would be the intrinsic factor.

Since the xanthine oxidase preparations used in the above experiments consisted of crude homogenates of gastric tissue, it is probable that they contained

both enzyme and substrate. There has been no demonstration that xanthine oxidase is secreted or is other than one of the system of enzymes occurring in mucosal cell for the same reason that it exists in many other types of tissues. The basis for assuming the intrinsic factor to be xanthine oxidase is, therefore, subject to doubt. Further, it has previously been demonstrated that the activity of folic acid is not increased following incubation with gastric juice (179).

This question also arises: is there an enzyme, xanthopterin oxidase, distinct from the xanthine oxidase enzyme? From Kalckar's work, it appears that a separate xanthopterin oxidase enzyme exists. He first reported an inhibitory effect by folic acid upon its action (194), but later demonstrated that 6-pteridylaldehyde, an irradiation breakdown product of folic acid, was the inhibiting agent (195).

Norris and Krebs state that there is no pterin oxidase, but that the xanthine oxidase enzyme acts upon xanthopterin (196). Reaction rate studies indicate that xanthine is the true substrate, and that xanthopterin forms a less dissociable complex with the enzyme, and thus in effect will actually exhibit an inhibitory action upon the oxidation of xanthine.

Relations of the substances therapeutically active in macrocytic anemias to the physiological and biochemical alterations associated with these anemias when untreated. Plasma cholinesterase activity. The reduced red blood cell and plasma cholinesterase level found in pernicious anemia has been mentioned. After remission, the esterase activity values return to normal. Davis (197), using dogs, was able to produce a macrocytic anemia by the administration of choline chloride or acetylcholine bromide. This, together with the above information led him to hypothesize that the mechanism responsible for relapse

in pernicious anemia was the increased acetylcholine concentration in the blood stream resulting from decreased cholinesterase activity.

Administration of diisopropyl fluorophosphate (DFP) suppresses cell and serum cholinesterase activity. Through repeated injections of this suppressant, low red blood cell and plasma cholinesterase activities have been maintained in man (198), and animals (199). No changes appeared in hepatic, renal or hematological functions. It was possible also by administering diisopropyl fluorophosphate to pernicious anemia patients to retain the reduced cell and plasma cholinesterase activity, even though complete remissions were induced with liver extract and folic acid therapy (200). These experiments make Davis' hypothesis untenable.

Incubation of folic acid with serum of low cholinesterase activity has been reported to result in an increased esterase content (197). Similarly the administration of folic acid to a normal individual causes the serum cholinesterase activity to increase. A direct effect of folic acid upon the enzyme is thus indicated. Since liver extracts (containing no folic acid) administration in pernicious anemia cases results in the same (201) cholinesterase activity increase as is produced by folic acid, these questions are raised, (a) does vitamin B_{12} cause an increase in the cholinesterase serum content of normal individuals, and (b) does incubation of vitamin B_{12} with esterase deficient serum result in an increased esterase activity? That is, does vitamin B_{12} also appear to affect this enzyme system directly, or is its action an indirect one, that of making folic acid available?

One would expect a similar low serum esterase activity in the other types of macrocytic anemia, particularly those in which folic acid appears to function

specifically. As yet few such studies have been made and there is an insufficiency of data to draw any conclusions concerning this possibility.

Serum cholesterol lowering. The lowering of serum cholesterol should be kept in mind because of its possible connection with the altered fat metabolism and the development of the neurological lesions of pernicious anemia. The presence of a so-called acetate-replacing factor in liver has been reported (202). This fact, coupled with the fact that acetate plays an important role in cholesterol synthesis, suggests a possible relationship of this factor to the pernicious anemia syndrome which should not be overlooked. Restoration of normal cholesterol values follows remission induced by liver extract therapy. Because of the inadequacy of folic acid in preventing the progression of neurological symptoms, it would be of interest to know whether folic acid has any effect on the serum cholesterol level.

Tyrosine metabolism. An increase in the excretion of phenolic compounds similar to that occurring in pernicious anemia takes place in the scorbutic guinea pig (203). In the former case, remission of the anemia is accompanied by a decrease in the excretion of phenolic compounds. This led to the observation that administration of folic acid (204) or folic acid-free liver extracts (205) accomplished a reduction in the amount of phenolic compounds excreted by the guinea pig comparable to that occurring after the administration of ascorbic acid.

There is thus a question as to whether or not ascorbic acid has a direct function in pernicious anemia etiology. The mean plasma level of ascorbic acid was found to be decreased in a series of pernicious anemia cases despite the intake of diets containing adequate amounts of the vitamin (206). However,

this lowered plasma level of ascorbic acid was found to be independent of the extent of the anemia and appears rather to be a function of the degree of achlorhydria. On the other hand, it was observed during World War II when there was a widespread vitamin C deficiency among the English people that the number of cases of pernicious anemia failing to respond to liver extract therapy was rising. Following the daily administration of vitamin C, however, these cases responded in a typical manner to liver extract (207).

Lactobacillus lactis Dorner ATCC 8000, (208), Lactobacillus leichmannii ATCC 4797 (169), and Lactobacillus leichmannii ATCC 313 (168) require the erythrocyte maturation factor for growth. It was found, however, that this requirement could be entirely replaced by the addition of ascorbic acid to the medium (209). Whether the effect of ascorbic acid is a specific one or is merely an agent affecting the redox potential remains to be decided. Sodium thioglycollate can be used to lower the redox potential, and does produce a growth response in these microbiological tests, but the effect is not as satisfactory as that produced by the ascorbic acid.

Porphyrin metabolism. That porphyrin metabolism is altered during pernicious anemia is apparent. Just where and why the alteration takes place remain unknown. Following folic acid therapy in pernicious anemia patients a red porphyrin-like pigment was excreted within four hours after the administration of the compound. No similar effect was noted following folic acid administration to normal individuals (210). Microbiologically, folic acid was found to exert an effect on the porphyrin production of Corynebacterium hoffmannii. Potassium cyanide diminishes porphyrin production, and also causes a slight decrease in growth (but the porphyrin production was measured in cultures

of the same optical density to prevent any effect due to an unequal amount of growth). The addition of folic acid to the culture counteracted the inhibitory effect of cyanide. In culture medium to which no cyanide had been added, the addition of folic acid caused an acceleration of growth (211).

More direct evidence concerning the effect of folic acid on porphyrin metabolism has been obtained using weanling rats raised on a folic acid deficient diet. The addition of folic acid to the diet resulted in an increased fecal porphyrin excretion, but no alteration in the growth rate. However, when 3 percent sodium benzoate was added to the rats' diet, it caused a decrease in porphyrin excretion, a decreased weekly weight gain, and a reduction in the number of white blood cells. Then when folic acid was added along with the sodium benzoate, the porphyrin excretion, growth rate, and the white blood count increased again. Glycine (2 percent) added in place of folic acid yielded similar results except that the white blood count remained low (212).

Glycine has been shown to act as a detoxifying agent for sodium benzoate. Also its utilization in porphyrin synthesis has been demonstrated. Thus the results of the above experiment can best be interpreted by assuming that folic acid is involved in the cellular production of glycine.

Biosynthesis and utilization of purines and pyrimidines. Through the use of microbiological techniques a relationship has been detected between the biosynthesis and utilization of purines and pyrimidines and the functions of folic acid and the erythrocyte maturation factor.

Thymine or thymidine can be substituted for folic acid when growing Streptococcus faecalis R. To obtain maximum growth with either folic acid or thymine it was necessary to add adenine to the culture medium. Three other species of folic acid requiring Lactobacilli were tested and shown to have the

same alternative requirements (165).

Competitive analogue-metabolite growth inhibition using methylfolic acid showed that folic acid functions in the synthesis of purines, pyrimidines or their equivalent (166).

What direct relationship exists between p-aminobenzoic acid and folic acid and between these two vitamins and the purines and pyrimidines is not definitely known. From studies using competitive analogue-metabolite growth inhibition, it appears that folic acid is a product of p-aminobenzoic acid metabolism. On the basis of the antibacterial index changes the following is proposed as the order in which the synthesis of essential factors become limited (213): (a) methionine, (b) purines and pyrimidines, (c) serine, (d) folic acid and (e) unknown substances related to amino acids. The order of folic acid and serine may be reversed. The question arises: does p-aminobenzoic acid function in the synthesis of purines and pyrimidines apart from its role in folic acid production?

When Escherichia coli are grown under conditions of sulfa inhibition for which purines and pyrimidines are the limiting factors for growth, an amine, 5(4)-amino-4(5)-imidazolecarboxamide, is found to accumulate in the culture medium (214). By the addition of a single carbon unit to this amine the purine ring structure is obtained. It would appear that a coenzyme functioning in the transfer of single carbon units has been blocked.

It has been indicated that there is a relationship between purines and vitamin B_{12}. When B_{12} was supplied to Lactobacillus lactis Dorner, purines were found to be non-essential although they are capable of exerting a sparing effect on the vitamin B_{12} requirement. Lactobacillus leichmannii ATCC 4797, which can also be used to assay for vitamin B_{12}, was found to also require

folic acid. Thymine after a lag phase was able to replace the folic acid (215).

Experiments with animals have indicated that dietary guanine and cytosine are not incorporated into the nucleic acids, though apparently adenine may be so utilized (216, 217). An inability to utilize purines and pyrimidines as effectively as the nucleosides in nucleic acid synthesis has been noted for a strain of Neurospora (218). The purine and pyrimidines requirements of many micro-organisms, however, indicates their ability to incorporate the preformed molecules into nucleic acids.

The replacement of the vitamin B_{12} requirement for micro-organisms by desoxyribosides has been reported (168, 169, 170). Clinical trials of the desoxyribosides in pernicious anemia, however, have apparently not been successful. The amount given or the method of administration may, however, have been unsuitable. At present the difficulty entailed in obtaining pure material seems to have prevented the completion of an adequate investigation. Until such an investigation is completed, no definite statement can be made concerning the effectiveness of desoxyribosides in pernicious anemia.

The nutritional replacement of folic acid by thymine, and of vitamin B_{12} by desoxyriboside in micro-organisms suggests an interesting clinical relationship that might exist. Since folic acid or thymine will cause a hematopoietic response in pernicious anemia, and whereas B_{12} is effective both hematologically and neurologically, it would be interesting to know if desoxyribosides are completely ineffective, or whether they might not cause a neurological response.

An impairment in the synthesis of desoxyribonucleic acids, but not of ribonucleic acids was shown to result when Lactobacillus casei was grown in a medium deficient in folic acid (219). To eliminate the possibility that

this effect on desoxyribonucleic acid synthesis was due to a specific vitamin deficiency, and not something which might result as a lack of any one of the nutritionally required B vitamins, a similar series of tests were carried out using media deficient in riboflavin or biotin. In these cases the only noticeable change was a moderate increase in the synthesis of both types of nucleic acids. Supplementation of the folic deficient Lactobacillus casei medium with an excess of thymine also resulted in an increase in both types.

The effect of folic acid in nucleic acid synthesis is also indicated in experiments on the sulfonamide inhibition of the psitacossis virus (a nucleoprotein)(220). Folic acid was found to effect a non-competitive reversal but p-aminobenzoic acid or pteroic acid were found to be competitive reversing agents. Glutamic acid had no effect upon the sulfonamide inhibition.

p-Aminobenzoic acid has been found to have an inhibitory effect on the development of Rocky Mountain and related ricketsial fevers (221, 222). A similar chemotherapeutic effect has been noted for p-aminobenzoic acid in typhus and murine typhus (223, 224). In view of the sulfonamide inhibition experiments upon the psitacossis virus it is difficult to deduce a possible explanation for this action of p-aminobenzoic acid unless it is acting as an inhibitor of a system in which a p-aminobenzoic acid derivative is an essential coenzyme.

SECTION II

EXPERIMENTAL RESULTS

Before the experimental information which was summarized in the preceding section can be logically explained, a number of questions must be answered. Our interest lay in exploring for leads which might aid in answering these fundamental questions.

1. What is the nature of the intrinsic factor?
2. What is the nature of the extrinsic factor?
3. What is the character of the reaction which occurs when the intrinsic and the extrinsic factors interact and where does this take place?
4. Does the erythrocyte maturation factor present in the liver develop
   a. as the direct result of a reaction between the intrinsic and the extrinsic factors which yields the erythrocyte maturation factor?
   b. from an initial elaboration of an intermediate product (the product of an intrinsic-extrinsic reaction) which is subsequently converted to the erythrocyte maturation factor?
   c. due to a more indirect action of the intrinsic factor (e. g., by facilitating absorption of the extrinsic factor, or perhaps by protecting the extrinsic factor from destruction in the stomach or small intestine)?
5. What is the function of the erythrocyte maturation factor?

The hypotheses that were proposed and tested are reported in this section.

A.

DISTRIBUTION OF FOLIC ACID ACTIVITY VS. THE MORPHOLOGICAL STRUCTURE OF INTESTINAL MUCOSA

The first hypothesis to be tested was one concerning folic acid and a particular type of cells occurring in the gastro-intestinal tract, the argentaffine cells. Folic acid in large doses had been shown to produce a hematopoietic response in pernicious anemia patients (but at that time had not yet been shown to be inadequate in the treatment of the neutral conditions). Jacobson, in 1939 while making a (225, 107) study of the argentaffine cells, noted the presence within these cells of granules having properties of pterins, and later, on the basis of absorption studies (226), suggested they were xanthopterin. There was a marked diminishment or complete absence of these cells in the alimentary tract of individuals who had had pernicious anemia, when they were compared to speciments obtained from other autopsy cases. Since it had been established that pernicious anemia results from the loss of a secretory capacity, a possible relation of these cells to the secretory function was indicated. Hence it was postulated that these cells could be:

a. the source of the intrinsic factor,
b. the site of localization of the intrinsic-extrinsic reaction product following its absorption, or
c. the source of a third or intestinal factor, whose production is somewhat dependent upon the intrinsic factor.

The non-identity of folic acid and the extrinsic factor had been established, but, since there had been demonstrated a functional relationship between these two factors, it seemed propitious to investigate whether the

pterin-like granules of the argentaffine cells might not in reality be composed of folic acid or some functional derivative.

Fresh hog duodenum was obtained from the local abattoir and separated by the method described by Jacobson into three fractions having the composition indicated:

a. a fraction almost entirely constituted of villi and the crypts of Lieberkuhn but containing the argentaffine cells,

b. a fraction predominantly Brunner glands, and

c. the duodenal musculature

Each fraction was assayed for its folic acid content microbiologically. The extracts were prepared by treating the tissues described in the general method for the enzymatic liberation of B-vitamins (227). Extracts were also prepared by using an enzyme concentrate (prepared from hog kidney)(151) which contained vitamin B_c conjugase, the enzyme which hydrolyzes the polyglutamyl derivatives of folic acid. Both types of preparations were assayed in both the Streptococcus faecalis R and Lactobacillus casei E methods (228, 229). No significant differences in the "microbiological folic acid activity" of the three layers could be demonstrated.

Subsequently Wilkinson (230) showed that only the first fraction from such a separation possesses clinical activity when used in the oral therapeutic treatment of pernicious anemia.

Our results indicated that there was no extensive localization of folic acid in the therapeutically active layer and they did not offer any substantiation to the possibility of the pterin-like granules' being folic acid.

TABLE I

FOLIC ACID CONTENT OF DUODENAL FRACTIONS

(micrograms per gm. moist tissue)

Lactobacillus casei Assay

	No Conjugase	Conjugase
Fraction a.	0.3	0.3
Fraction b.	0.57	0.85
Fraction c.	0.24	0.35

Streptococcus faecalis R Assay

	No Conjugase	Conjugase
Fraction a.	0.45	0.8
Fraction b.	0.75	1.35
Fraction c.	0.45	0.8

B.

THE EFFECT OF THE INTRINSIC FACTOR UPON THE INHIBITORS OF FOLIC ACID CONJUGASE

When it was found that pernicious anemia patients, unlike normal subjects, could not utilize folic acid conjugates (181, 182), the intrinsic factor was tested for its activity as a conjugase enzyme. It was found to have no direct action upon folic acid conjugates. However, since conjugase inhibitors had been demonstrated to be present in natural materials containing folic acid conjugates (183), we attempted to show that the intrinsic factor might have an indirect action on the utilization of folic acid conjugates, that is, that the intrinsic factor acts as a counter-inhibitor and in some way prevents the action of the naturally occurring inhibitors. Accordingly, yeast extracts (which had been shown to contain both folic acid conjugates and the conjugase inhibitor) were incubated with a conjugase preparation in the presence and absence of gastric juice. It was proposed that if the intrinsic factor exerted its action by counteracting the conjugase inhibitor, an increased amount of free folic acid should be liberated from the samples to which intrinsic factor (neutralized gastric juice from a normal subject) had been added. Microbiological assays for folic acid activity using both Streptococcus faecalis R (228) and Lactobacillus casei (229) were carried out on the various incubation samples, but no effect of the added gastric juice could be detected. This postulated mechanism was therefore discarded.

TABLE II

FOLIC ACID LIBERATION FROM ITS CONJUGATES

(micrograms per gm. yeast extract)

Incubation Mixture	Folic Acid Content — Assay Organism	
	Lactobacillus casei	Streptococcus faecalis R
Yeast Extract (dry weight)	0.9	1.1
Conjugase preparation (0.05 cc.)	0.0	0.0
Neutral gastric juice (0.1 cc.)	0.0	0.0
Yeast Extract + Conjugase preparation (0.1 mg - 0.1 cc.)	17.8	4.9
Yeast Extract + Neutral gastric juice (0.1 mg - 0.2 cc.)	0.9	1.1
Yeast Extract + Neutral gastric juice + Conjugase preparation (0.1 mg - 0.2 cc. - 0.1 cc.)	17.8	4.9

The presence of xanthopterin has been shown to increase the yield of free folic acid when liver homogenates are incubated. This is not the result of its conversion to folic acid as was first thought but is probably the result of its ability to inhibit an enzyme system which destroys folic acid (136). It is conceivable that xanthopterin might likewise interfere with the naturally occurring inhibitors which limit the activity of the liver conjugase. Similarly, one could postulate such a function for the argentaffine cells when they produce xanthopterin or a xanthopterin derivative. Hence, xanthopterin was used in a series of tests similar to those described in the preceding paragraph but it was found to have no appreciable effect on reactivating the inhibited conjugase system.

TABLE III

THE EFFECT OF XANTHOPTERIN UPON

THE LIBERATION OF FOLIC ACID FROM ITS CONJUGATES

Incubation Mixture	Assay Organism *Streptococcus faecalis* R
Yeast Extract (dry weight)	0.45
Xanthopterin (10 γ)	-
Conjugase (0.05 cc.)	-
Yeast Extract + Conjugase preparation (0.1 mg - 0.1 cc.)	3.3
Yeast Extract + Xanthopterin (0.1 mg - 100 γ)	0.4
Yeast Extract + Xanthopterin + Conjugase preparation (0.1 mg - 100 γ - 0.1 cc.)	2.4

C.

THE RELATIONSHIP OF XANTHINE OXIDASE TO THE INTRINSIC FACTOR

There are several indications that folic acid is not the form of the vitamin, which is eventually utilized; thus, there is an extreme discrepancy between the dietary intake and excretion of folic acid especially when a comparison is made with the other B-vitamins, and there are a large number of derivatives which have been found to occur naturally which have varying activities depending upon the test organism used. Because of this there have been a number of attempts to produce a more active form of folic acid. Jacobson and Good (231) reported that by the incubation of folic acid with a xanthine oxidase preparation (from liver), they obtained a product possessing

a hematological activity greater than could be accounted for on the basis of its folic acid content. This report ushered in a series of experiments concerned with the action of xanthine oxidase upon folic acid. The work of Kalckar and Klenow (232) indicated that there was an enzyme, xanthopterin oxidase, distinct from xanthine oxidase, but as yet there does not seem to be any clear cut evidence as to whether or not this is a separate enzyme rather than just another manifestation of a non specific xanthine oxidase-like activity. The term xanthine oxidase will be used to designate the preparation which was used in the work reported below and which catalyzed the oxidation of both the substrates (xanthine and xanthopterin).

At first it was thought that folic acid inhibited the activity of this enzyme, but later it was shown that 6-pteridyl aldehyde, a breakdown product of folic acid formed upon illumination, was the true inhibiting agent. There was no discernable difference in the ultra violet absorption spectra of folic acid after being treated with the enzyme, nor could any alteration in its microbiological activity be detected (194). Norris and Majnarich (133), however reported that the activity of folic acid when tested upon bone marrow cultures is very much increased after being treated with a xanthine oxidase (milk) preparation. These investigators also used gastric mucosa and demonstrated it to possess the folic acid activating activity of their xanthine oxidase preparations. From this observation they postulated the identity of xanthine oxidase with the intrinsic factor. We were thus interested in making a direct demonstration of the presence in gastric juice of xanthine oxidase.

Using the decolorization of methylene blue in Thunberg tubes as our criterion of oxidase activity, we found that normal gastric juice showed no oxidase activity for any of the substrates tested. Gastric mucosa slowly

decolorized methylene blue even in the absence of added substrate, presumably due to the presence of other dehydrogenating enzymes and their corresponding substrates. We were unable to demonstrate any pterin oxidase activity in the secretory products of gastric mucosa. Furthermore, we were unable to obtain any indication that folic acid could function as a substrate for an enzyme preparation known to possess xanthopterin oxidase activity.

The enzyme preparations that were used were:

a. xanthopterin oxidase prepared from raw milk by the method of Kalckar and Klenow (232).

b. gastric juice from a normal subject (filtered and neutralized).

c. the supernatant fluid obtained by centrifuging an extract made by homogenating mucosa scraped from the stomach of a rat with ten volumes of 0.2 M phosphate buffer (pH 6.8).

The substrates that were used were:

a. xanthopterin, 100 micrograms per ml. with enough sodium bicarbonate added to affect solution of the pterin.

b. folic acid, 100 micrograms per ml. of phosphate buffer, 0.2 M., pH 6.8.

One and a half ml. of the indicated enzyme sources were tipped, after temperature equilibration at 37°C., into a solution containing 2 ml. of the substrate and 1 ml. of a 0.01 percent solution of methylene blue.

TABLE IV

ACTIVITY OF THE INTRINSIC FACTOR UPON FOLIC ACID IN AN OXIDASE SYSTEM

Enzyme Source	Substrate		
	No addendum	Xanthopterin (200γ)	Folic Acid (200γ)
Xanthopterin oxidase from milk	No change	Decolorized within one hour	No change
Gastric juice	No change	No change	No change
Gastric mucosa	Decolorized overnight	Decolorized overnight	Decolorized overnight

D.

STUDIES USING BONE MARROW CULTURES

Without an assay specific for the erythrocyte maturation factor it is impossible to characterize either the reaction by which it is formed or to locate the site of formation. At the time this investigation was undertaken the only available assay method for this factor was a clinical trial upon pernicious anemia patients in relapse.

The inadequacies of this technique are very apparent. The investigator is faced with what might be termed "human factors" a reticence to experiment when a human life is involved; difficulty in obtaining subjects; individual variation in response, usually overcome in animal experimentation by using a statistical approach; and the possibility of false results due to a spontaneous remission of the patient. In addition, there are the factors of time and expense, both of which are great. The desirability of obtaining a simple, concisive method of assay is obvious. Attempts to produce a syndrome in

animals similar to the pernicious anemia syndrome in man have consistently failed. Wherever positive results were indicated in such attempts, it was always found that some other nutritional factors, in addition to the erythrocyte maturation factor, would cause a remission of the induced anemia. The small amount of data concerning the chemical nature of the erythrocyte maturation factor precluded the development of a chemical test.

In vitro studies based upon the clinical findings have been attempted with bone marrow. It is possible to obtain a relatively good concentration of reticulocytes from bone marrow by centrifugation as these cells have a smaller density. than most of the bone marrow elements. A report by Hay (233) indicated that the maturation of reticolcytes incubated in vitro in a glucose-free Tyrode's solution could be effected by the presence of the erythrocyte maturation factor. We experimented in an attempt to utilize the method on a quantitative basis. Uniform suspensions of reticulacyte preparations concentrated from rat bone marrow by centrifugation (233) were incubated in glucose-free Tyrode's solution at 37°C. for periods of four and six hours with varying amounts of liver extract (Eli Lilly and Co., 10 U.S.P. units per ml.). Smears were made before and after incubation and the reticulocyte counts compared. We were unable to show anything other than a qualitative indication that the addition of liver extract caused an increase in the maturation of the reticulocytes.

E.

DEVELOPMENT OF ASSAY METHODS

Since progress on studies attempting to interrelate the hematopoietic agents was drastically limited by the non-existence of any practical assay method for the erythrocyte maturation factor, it was decided that a search for

a simple method for analyzing for the biological activity associated with this factor would be a worthwhile project. Consequently, such a search was undertaken.

The use of microorganisms for asssying the various known components of the B-vitamin group has been extensively developed. It is now generally believed that all of the B-vitamins are essential cell constituents of all microorganisms as well as other types of cells. For each organism it is possible, however, to place each of the vitamins into one of four catagories: "non-essential" vitamins which can be synthesized at an adequate rate by the organism itself; "essential" vitamins which must be supplied intact to the organism; vitamins that can be synthesized when one or more parts of the vitamin are supplied; and those vitamins that are synthesized so slowly that they can become the factor limiting the rate of growth.

It seemed feasible, therefore, that it might be possible to obtain a microbiological assay for the erythrocyte maturation factor if it were a catalyst of general biological importance. It appeared likely that p-aminobenzoic acid was fundamentally involved either in the utilization, function, or elaboration of the erythrocyte maturation factor, because (a) p-aminobenzoic acid is a part of the folic acid molecule and (b) folic acid is involved in hematopoiesis, but is not identical with the erythrocyte maturation factor. (It is uncertain whether p-aminobenzoic acid and folic acid function by being incorporated into the same coenzyme).

Since folic acid (also thymine) and the erythrocyte maturation factor can to some extent be interchanged in the treatment of the hematological symptoms in pernicious anemia, it was decided to see if similar substitutions could be made in the requirements of microorganisms. If such cases could be demonstrated

then microbiological assays could be devised. Since it was not known whether p-aminobenzoic acid functions solely in the synthesis of folic acid coenzyme(s) or is in addition incorporated into other coenzymes, it was decided that a wider range of possibilities could be encompassed by considering p-aminobenzoic acid functions rather than folic acid functions. Two alternatives were considered: the complete replacement of the p-aminobenzoic acid requirement by the addition to the media of sources of the erythrocyte maturation factor, and supplementation suboptimum amounts of p-aminobenzoic acid with the erythrocyte maturation factor (i. e., use the latter as a sparing agent). In carrying out these studies, it was of course necessary to test the influence of purines, pyrimidines, folic acid, and individual amino acids (substances which had been shown to be functionally related to p-aminobenzoic acid)(213) since the nutritional requirement for them might be replaced by utilization of the erythrocyte maturation factor, or they conceivably could be functionally related by reason of their being metabolites necessary for the functioning of the erythrocyte maturation factor.

Preliminary tests were made using three p-aminobenzoic acid-requiring organisms: Lactobacillus arabinosus, Neurospora crassa mutant, and Acetobacter suboxydans. Due to the difficulty experienced in obtaining growth of the last organism in a well characterized media, it was soon dropped from the experiments to be described.

From a study of the substitutions which could be made in the nutritional components furnished these organisms, it was possible to demonstrate a biological response to several factors which are present in liver extracts used therapeutically but which could not be identified with known vitamins or growth factors.

Factor replacing purines for Lactobacillus arabinosus. This organism requires for growth, in addition to other substances, (a) a purine, (b) a pyrimidine, and (c) p-aminobenzoic acid. However, it was found that the purine requirement can be replaced by some substance(s) present in liver extracts which, on the basis of the small amounts of very crude concentrates required, must be active at a level very much lower than that at which the purines must be supplied. The assay procedure developed for this factor is summarized in Table VI and the typical response of the organism to it is recorded. In an investigation of some of the chemical and physiological properties subsequently undertaken by my colleague, Mrs. Robert Lane, it was shown that this factor(s) differed from the purines themselves not only in the magnitude of the requirement but in a number of chemical and physical properties (234).

Pyrimidine replacing factor for Lactobacillus arabinosus. In a similar fashion it was shown that the pyrimidine requirements of this organism could be met by something present in liver extracts, and an analogous assay method was devised (see Table VII for procedure and characteristic response of the organism). This factor was believe to be an entity distinct from the purine-replacing factor because the substances active in the two tests did not behave similarly when liver extracts were fractioned by absorption on and elution from charcoal and by partition between aqueous and organic solvents (234).

Two factors sparing the p-aminobenzoic acid requirement of Neurospora crassa. The p-aminobenzoic acid requiring mutant of Neurospora crassa apparently requires p-aminobenzoic acid itself and it is impossible to replace the vitamin by the amino acids, purines, and pyrimidines, whose syntheses it catalyzes, nor even by crude liver extracts. However, if suboptimal amounts

of p-aminobenzoic acid, insufficient to promote appreciable growth (0.002 micrograms per 10 ml. culture) are supplied, then the mold responds to substances present in crude liver extracts.

Liver extracts (Eli Lilly and Company) having potencies of 2, 5, 10, and 20 U. S. P. units per ml. were tested according to the procedure outlined in Table VIII. These were diluted to contain equal unitage per cubic centimeter. When assayed, however, unequal responses were obtained; on a unit basis, the 5, 10, and 20 unit extracts were less active than the less refined 2 unit preparations. Purines, pyrimidines, folic acid and hydrolyzed casein were tested for their ability to replace the stimulatory material(s) in 2 unit liver preparations. Their combined effect was not comparable to that obtained with the 2 unit preparations. An electrolytic fractionation of the 2 unit liver extract was carried out in a five cell unit (235). Upon recombination of cups I and V, the most basic and the most acidic fractions, almost all the original activity was recovered. However, when tested separately these fractions showed only a fraction of the activity of the starting material.*

The acidic fraction (that in cup V) was tried as a supplement to the 5, 10, and 20 unit preparation in assays. Increased growth response was indicated but consistent results could not be obtained. Conversely, in the presence of an addendum of the basic fraction, the response was a measure of the "acid factor".

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* Subsequently, when highly refined concentrates of erythrotin (vitamin B_{12}) and folinic acid (a folic acid derivative required by Leuconostoc citrovorum) became available it was found that in the presence of the suboptimal amount of p-aminobenzoic acid, vitamin B_{12} was relatively inactive; folinic acid concentrates gave some growth response; the combination of vitamin B_{12} and folinic acid gave a better response, but one that was not equivalent to that obtained when 2 unit liver extracts were used.

TABLE V

RESPONSE OF A P-AMINOBENZOIC ACID REQUIRING MUTANT OF NEUROSPORA CRASSA (ATC. NO.

Medium, testing conditions, etc., those described in Table VIII.

	Growth Response in Arbitrary Visual Units	
	No p-amino-benzoic acid	0.02 γ p-amino-benzoic acid per 10 ml. culture
Correlation of growth response and therapeutic potency		
No addendum	−	+ −
8 milliunits of 2 U.S.P. unit liver extract	+	++++
8 milliunits of 10 U.S.P. unit liver extract	+ −	+
8 milliunits of 20 U.S.P. unit liver extract	+ −	+
Activity of fractions separated by electrical transport tested at a level equivalent to that obtained from 8 milliunits of 2 U.S.P. unit liver extract per 10 ml. culture		
Cup I (pH 10.3)	−	++
Cup II (pH 7.1)	−	+
Cup III (pH 6.2)	−	−
Cup IV (pH 5.25)	−	−
Cup V (pH 3.0)	−	+
All Cups	+ −	++++
Cups I and V	+ −	+++

TABLE V
(continued)

	Growth Response in Arbitrary Visual Units	
	No p-amino-benzoic acid	0.02 γ p-amino-benzoic acid per 10 ml. culture
Activity of liver concentrates in presence of acidic and basic factors separated by electrical transport equivalent to 8 milli-units per culture		
Acidic fraction supplement (Cup V)		
Alone	-	+
Plus 8 milliunits of 5 U.S.P. unit liver extract	±	++
Plus 8 milliunits of 10 U.S.P. unit liver extract	±	++
Plus 8 milliunits of 20 U.S.P. unit liver extract	±	++
Basic fraction supplement (Cup I)		
Alone	-	++
Plus 8 milliunits of 5 U.S.P. unit liver extract	±	+++
Plus 8 milliunits of 10 U.S.P. unit liver extract	±	+++
Plus 8 milliunits of 20 U.S.P. unit liver extract	±	+++

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F.

EVALUATION OF ASSAY METHODS

Other members of the research groups of The Biochemical Institute had concurrently developed microbiological assays for unidentified factors present in the liver preparations used in treating pernicious anemia patients. Four of these assays were based upon the activity of the naturally occurring substances in reversing the toxicity of growth inhibitors structurally related to p-aminobenzoic acid, folic acid, and a purine (guanine). Because these factors were active in reversing such inhibitors, they were assumed to be functionally related to p-aminobenzoic acid and folic acid and hence might be the erythrocyte maturation factor itself or a structurally related compound. In the surveys described below these four assays were run simultaneously with the three assays developed by the author. Also included in the screening program was the acetate replacing factor (202) since certain liver preparations were a rich source of this factor and since there is a functional relationship of this factor to the metabolism of cholesterol and fatty acids, both or either of which may be concerned in the neurological involvement in pernicious anemia.

Two approaches were used in attempting to establish which, if any, of these factors is the erythrocyte maturation factor: first, a comparison was made of the microbiological activities of a number of preparations having different anti-anemic (clinically) potencies and determining how closely the relative clinical and microbiological potencies paralleled one another. A more specific criterion for detecting a test which responded to the erythrocyte maturation factor would be the determination of the microbiological response to the incubation procedures which produce clinical results. That is, from the work of Castle and others described in detail in the first section there

is evidence for the formation of a heat labile compound intermediary in the formation of the erythrocyte maturation factor. It is plausible that such an intermediate could be utilized in lieu of the erythrocyte maturation factor by some organisms. Thus, if an enhanced activity could be demonstrated for an incubation mixture of intrinsic and extrinsic factor preparations in one of these assays it would be indicative that the organism might require the erythrocyte maturation factor or its precursor.

The preparations used in screening the various assay methods for uncharacterized factors were obtained in the following manner:

Extrinsic factor: A piece of weighed, lean beef was chopped and homogenized one half hour in a Waring blendor with a measured amount of water. To the homogenate was added enough 95 percent ethanol to yield solution having an alcohol content of 80 percent. This was allowed to stand with occasionally stirring for four hours and then stored overnight in the refrigerator. The mixture was filtered (with aid of Filter-Cel) and evaporated on a steam bath until it became very syrupy. This concentrate was extracted twice with benzene and twice with ether. After the amount of solid material had been determined, the preparation was diluted with water (50 mg. solids per ml.), the solution tubed in five ml. aliquots, and the samples sterilized and stored in the refrigerator.

Intrinsic factor: Two sources were used; (a) undiluted gastric juice (obtained by lavage of the author) which was immediately neutralized and stored in the frozen state, (b) a dried preparation of hog intestinal mucosa (Eli Lilly and Company).

Erythrocyte maturation factor: Commercial preparations (Eli Lilly and Company) of liver extracts (Liver Injection U. S. P.) of varying degrees of

refinement indicated were used.

Incubation mixtures: One ml. aliquots of the extrinsic preparations were incubated at 37° C. for four hours with 1.5 ml. of neutralized gastric juice, and the samples then diluted for assay. One ml. aliquot of water plus 1.5 ml. of the normal gastric juice were carried through the procedure simultaneously and served as controls.

Tables VI through XII summarize the assay methods and record the results of tests which were typical of a number of trials.

TABLE VI

PURINE REPLACING FACTOR

Organism: Lactobacillus arabinosus 17-5
Medium: Medium of Landy and Dicken (236); asparagine, folic acid and purines omitted. 100 millimicrograms p-aminobenzoic acid and 100 millimicrograms folic acid added per tube.
Testing procedure: 16 hour incubation at 30° C. - 10 cc. cultures

Addendum	Growth Response in Galvanometer Deflection
Micrograms each of guanine and adenine	
0	15
0.1	30
0.2	39
0.5	44
1.0	46
2.0	52
2.5	55
Milliunits of 20 U.S.P. unit liver extract	
0	15
0.4	20
0.8	24
1.6	55
4.0	68
8.0	68
Milliunits of 5 U.S.P. unit liver extract	
0	14
0.4	18
0.8	20
1.6	34
4.0	49
8.0	67
Milliliter of neutral gastric juice	
0.	15
0.003	18
0.006	18
0.015	21
0.03	20
0.06	27

TABLE VI
(continued)

Addendum Milligrams of extrinsic factor preparation	Growth Response in Galvanometer Deflection
0	16
0.01	26
0.02	28
0.05	34
0.1	49
0.2	63
Incubation mixture containing indicated milligrams of extrinsic factor preparation	
0	19
0.01	27
0.02	26
0.05	48
0.1	51
0.2	70

TABLE VII

PYRIMIDINE REPLACING FACTOR

Organism: Lactobacillus arabinosus 17-5
Medium: Medium of Landy and Dicken (236); asparagine, folic acid and pyrimidines omitted. 100 millimicrograms p-aminobenzoic acid and 0.1 micrograms folic acid per tube.
Testing procedure: 16 hours incubation at 30° C. - 10 cc. cultures

Addendum	Growth Response in Galvanometer Deflection
Micrograms of uracil	
0	17
0.1	53
0.2	61
0.5	66
1.0	73
2.0	76
Milliunits of 20 U.S.P. unit liver extract	
0	10
5	17
10	17
20	30
50	66
100	68
Milliunits of 5 U.S.P. unit liver extract	
0	14
5	17
10	22
20	24
50	49
100	65
Milliliter of neutral gastric juice	
0.001	32
0.003	35
0.006	32
0.015	33
0.03	32
0.06	32

TABLE VII
(continued)

Addendum Milligrams of extrinsic factor preparation	Growth Response in Galvanometer Deflection
0.005	36
0.02	38
0.05	44
0.1	49
0.2	60
0.5	74
Incubation mixture containing indicated milligrams of extrinsic factor preparation	
0.005	31
0.02	33
0.05	37
0.1	40
0.2	49
0.5	62

TABLE VIII

FACTOR STIMULATING THE GROWTH OF NEUROSPORA CRASSA
(p-aminobenzoic acid requiring mutant)

Organism: Neurospora crassa ATC 9278.
Cultures inoculated with a dilute saline suspension of spores scraped from agar stock cultures.

Medium:

Ammonium tartrate	5.0 g.
Ammonium nitrate	1.0 g.
Potassium monobasic phosphate	1.0 g.
Magnesium sulfate	0.5 g.
Calcium chloride	0.1 g.
Sodium chloride	0.1 g.
Sucrose	15.0 g.
Biotin	5.0 γ
Trace Elements	1.0 cc.
Water to make 1 liter	

Trace Elements:

Sodium tetraborate	88 mg.
Ammonium molybdate	64 mg.
Ferric chloride	500 mg.
Zinc sulfate heptohydrate	2 g.
Cupric chloride	270 mg.
Manganous chloride	45 mg.
Water to make 1 liter	

Medium for each 10 ml. culture supplemented with the acidic fraction separated by electrolysis (Cup V, Table V) from 8 milliunits of Eli Lilly and Co. 2 U. S. P. unit liver preparations.

Testing Procedure: ~~24~~ 72 hour incubation at 30° C. - 10 cc. cultures in 50 cc. Erlenmeyer flasks. No shaking.

Addendum Milliliter of neutral gastric juice	Growth Response in estimated visual units
0.01	+
0.02	+
0.05	+
0.1	+

* Check and correct

TABLE VIII
(continued)

Addendum Milligrams of extrinsic factor preparation	Growth Response in estimated visual units
0.005	+++
0.05	+++
0.1	+++
0.2	+++
Incubation mixture containing indicated milligrams of extrinsic factor preparation	
0.005	++
0.05	+++
0.1	+++
0.2	++++

TABLE IX

ANTI-AZOGUANINE FACTOR*

Organism: Lactobacillus arabinosus 17-5
Medium: Medium of Landy and Dicken (236); guanine omitted. 0.1 γ p-amino benzoic acid, 0.1 γ folic acid, and 5 γ azoguanine added per culture.

Testing procedure: 18 hours incubation at 30° C. - 10 ml. cultures.

Addendum Micrograms of guanine	Growth Response in Galvanometer Deflection
0	2
2	4
5	8
10	17
20	25
50	42
100	52
200	56
Milliliters of neutral gastric juice	
0.05	6
0.1	6
0.2	6
0.5	6
Milligrams of extrinsic factor preparation	
0	6
0.01	6
0.02	5
0.05	5
0.1	6
0.2	6
0.5	7
1.0	10

TABLE IX
(continued)

Addendum Incubation mixture containing indicated milligrams of extrinsic factor preparation	Growth Response in Galvanometer Deflection
0	5
0.01	6
0.02	6
0.05	6
0.1	7
0.2	6
0.5	10
1.0	14

The test for this factor(s) developed by Dr. Beverly Guirard, is based upon the counteraction of the inhibition resulting from the addition to the medium of a structural analogue of guanine, (7-amino-1-y-triazolo-(d)-pyrimidine, designated azoguanine). It is believed that the organism responds in this medium if either the guanine (or derivatives) required for nucleic acid metabolism is supplied or if a factor(s) catalyzing the synthesis of this purine is present in catalytic amounts.

TABLE X

ACETATE REPLACING FACTOR*

Organism: Lactobacillus casei
Medium: Medium of Landy and Dicken (236); sodium acetate omitted. 25 mgm. KH_2PO_4 added per culture.

Testing procedure: 18 hours incubation at 37° C. - 10 ml. cultures.

Addendum	Growth Response in Galvanometer Deflection
Micrograms of 30 U.S.P.	
0	15
5	14
10	21
20	42
50	59
Milliliters of neutral gastric juice	
0.001	15
0.015	18
0.03	9
0.06	11
Milligrams of extrinsic factor preparation	
0.005	16
0.05	11
0.1	17
0.2	13
Incubation mixture containing indicated milligrams of extrinsic factor preparation	
0.005	20
0.05	14
0.1	16
0.2	18

* The assay method reported here is essentially that used by Guirard, Snell and Williams (202) when they demonstrated the natural occurrence of factors which could affectively replace the acetate requirements of certain lactobacilli.

TABLE XI

METHYLFOLIC REVERSING FACTOR*

Organism: Leuconostoc mesenteroides 8293
Medium: Medium of Landy and Dicken (236); folic acid omitted. 10 micrograms of p-aminobenzoic acid, 30 micrograms of folic acid and 200 γ methylfolic added per culture.

Testing procedure: 18 hours incubation at 30° C. - 10 ml. cultures.

Addendum	Growth Response in Galvanometer Deflection
Micrograms of 30 U.S.P.	
0	5
2	8
5	14
10	20
20	51
50	69
Milliliters of neutral gastric juice	
0.001	10
0.015	9
0.03	11
0.06	16
Milligrams of extrinsic factor preparation	
0.005	10
0.05	9
0.1	10
0.2	20
Incubation mixture containing indicated milligrams of extrinsic factor preparation	
0.005	10
0.05	11
0.1	15
0.2	21

* This test, developed by Dr. Winifred Harding and Dr. William Shive, measures the activity of factor(s) reversing the inhibition of a structural analogue of folic acid. Subsequently Dr. Shive and his coworkers, using this assay isolated from the 30 unit liver extract the desoxyriboside, thymidine.

TABLE XII

SULFONAMIDE REVERSING FACTOR*

Organism: Lactobacillus arabinosus 17-5
Medium: Medium of Landy and Dicken (236); folic acid omitted. 10 mg. of sulfonamide, 3 γ of p-aminobenzoic acid, and 3 γ folic acid added per cultures.

Testing procedure: 18 hours incubation at 30° C. - 10 cc. cultures.

Addendum	Growth Response in Galvanometer Deflection
Micrograms of 20 U.S.P.	
0	14
2	11
5	20
10	34
20	37
50	63
100	83
200	89
Milliliters of neutral gastric juice	
0.001	15
0.003	15
0.006	17
0.015	20
0.03	25
0.06	24
Milligrams of extrinsic factor preparation	
0.005	27
0.01	72
0.02	87
0.05	87
0.1	90
0.2	95

TABLE XII
(continued)

Addendum Incubation mixture containing indicated milligrams of extrinsic factor preparation	Growth Response in Galvanometer Deflection
0.005	30
0.01	70
0.02	87
0.05	86
0.1	85
0.2	97

* This is one modification of several assay methods employing inhibition developed by Dr. William Shive and his associates in their search for factors functionally related to p-aminobenzoic and folic acids (213).

That none of the tests employed were responding specifically to a product formed by an extrinsic-intrinsic factor reaction was evident from the results of the screening program for which typical data were reported. However, the results of the comparison of the microbiological activity with clinical potency were not so conclusive. The main difficulty encountered was the number of accessory factors which were capable of altering the response of the organism. Thus, ideally, for a good microbiological assay, the medium should sustain little or no growth of the microorganism in absence of the growth factor, and make possible maximum growth in its presence. In addition there should be a regular increase in response with increasing increments of the growth factor until the maximum response is reached. The tests for the purine and pyrimidine replacing factors are clearly not of this type, i. e., they are unduly influenced by the presence of other substances occurring in the natural extracts which were assayed. Now that refined preparations are available of new growth principles, these tests might prove useful as an aid in the elucidation of their function in cell metabolism.

The Neurospora crassa mutant, in a medium supplemented with an acidic fraction of crude liver extract, appeared to be responding to liver preparations of varying degrees of refinement at concentrations corresponding to their therapeutic effectiveness, and hence was considered as a possible test organism for the erythrocyte maturation factor. However, it was realized that the preliminary method used would have to be refined and a quantitative procedure developed. Just at the time this was undertaken bacterial assays known to be specific for vitamin B_{12} became available so our search for a suitable assay organism was dropped in order to pursue the investigation on the extrinsic-intrinsic phenomenon described in the following section.

G.

ERYTHREIN AND APOERYTHREIN, AND THEIR RELATIONSHIP TO THE ERYTHROCYTE MATURATION FACTOR

We now know that none of the assay methods outlined in the previous section, with the possible exception of the one utilizing a Neurospora mutant, responded significantly to the erythrocyte maturation factor (erythrotin or vitamin B_{12}) and for this reason we did not detect any significant correlation between the microbiological activities and therapeutic effectiveness, nor any appreciable activation of extrinsic factor preparations by normal gastric juice. Subsequently, when other microbiological methods which did respond to vitamin B_{12} had been developed both here at the Biochemical Institute by Dr. Shive and his coworkers (213) and in other laboratories (208)(169), the intrinsic-extrinsic-vitamin B_{12} relationship was studied using these organisms.

We were very surprised to find that unheated normal gastric juice or extracts of gastro-intestinal mucosa completely inhibited the growth response elicited by erythrotin,* but that this inhibition could be overcome by adding an excess of the vitamin. Further exploration of this inactivation revealed that a protein-like fraction of normal gastric juice combines with erythrotin and renders it unavailable to microorganisms. The microbiological tests for vitamin B_{12} can, therefore, be easily adapted to quantitative assay methods for this principle.

The next table outlines the method we found to be most practical in testing for the erythrotin inactivating factor occurring in normal gastric

* The author is very grateful to Dr. Shive for his generosity in making available to her samples of his erythrotin concentrates before Vitamin B_{12} preparations could be obtained from any other source.

juice and hog intestinal mucosal extracts. The test organism is Escherichia coli, cultivated in a salts-glucose medium supplemented with vitamins. The growth of the organism is inhibited by the addition of sulfanilamide, 100 mg. per liter. The sulfanilamide hinders in some manner the catalytic action of p-aminobenzoic acid in one of the chemical reactions necessary for the ultimate synthesis of methionine. This inhibition can be overcome (1) by adding relatively large amounts of p-aminobenzoic acid or (2) by supplying the organism's requirement for methionine in the form of the amino acid itself, or (3) by supplying the organism with vitamin B_{12}. The assay for the inactivating principle (present both in normal gastric juice and extracts of dried hog duodenal mucosa) is carried out in a medium to which there has been added just enough erythrotin to overcome the sulfanilamide inhibition. Increments of the inactivating principle which tie up part or all of the erythrotin activity can be detected by the reduction in the growth response as is illustrated in the following table which records one of the initial experiments in which the phenomenon was first observed.

The union between erythrotin and the inhibiting principle is a thermolabile one as the gastric principle produces no inhibition if it is heated in solution at 100°C. either before or after it is mixed with erythrotin. Hence, the samples cannot be autoclaved, but are added unsterilized. We have encountered no difficulties with bacterial contamination in this relatively simple medium during the short incubation period. When the lactobacilli which respond to vitamin B_{12} are used, however, the gastric or mucosal samples must be sterilized by filtration to avoid contaminations which interfere with the assay. This procedure is not too satisfactory because some of the vitamin B_{12} binding activity is either absorbed or destroyed during the filtration. For this reason, as well as for reasons of

TABLE XIII

TYPICAL MICROBIOLOGICAL RESPONSES TO THE ~~ERYTHROTIN~~ Vitamin B_{12} BINDING FACTOR

Organism: Escherichia coli (saline suspension from 24 hour salts-glucose culture)

Medium: Salts-glucose medium suplemented with vitamins (213), and 200 mg. sulfanilamide per liter

Testing Procedure: 5 ml. samples containing aliquots of normal gastric juice unheated and heated (100°C., 10 minutes)
5 ml. of medium
Incubated at 37°C. for 15 hours

Addendum vitamin B_{12} Micrograms of ~~erythrotin~~	Growth Response in Galvanometer Deflection *
0	10
0	9
0.0001	14
0.0002	18
0.0005	26
0.001	34
0.002	36
0.005	38
Milliliters of normal gastric juice (unheated) plus 0.005 micrograms of ~~erythrotin~~ vitamin B_{12}	
0.2	22
0.5	15
1.0	14
2.0	10
Milliliters of normal gastric juice (heated) plus 0.005 micrograms of ~~erythrotin~~ vitamin B_{12}	
0.2	29
0.5	26
1.0	29
2.0	25

* Distilled water reads 0
Opaque filter reads 100

simplicity, the Escherichia coli test is the one of choice.

The formation of the microbiologically inactive complex effectively takes place at extreme dilutions (10^{-11}M) as is shown by the fact that the erythrotin does not even have to be incubated with the protein prior to the assay but can be mixed with the medium before the medium is added to the protein samples. The critical ratio of inhibitory factor to erythrotin is constant as is shown by the following table in which it can be seen that every three-fold increase in erythrotin requires a three-fold increase in the amount of inhibiting factor necessary to inactivate it. That the inhibition resulting from the presence of unheated gastric juice or hog mucosa can be overcome by addition of stoichiometric amounts of erythrotin rules out the possibility that the observed effect is the result of proteolytic or mucolytic activity of the inactivating principle.

TABLE XIV

RATIO OF INHIBITORY FACTOR TO ~~ERYTHROTIN~~ Vitamin B_{12}

Testing Procedure: The organisms, medium and cultural conditions described in Table XIII were used: the volume of the samples plus medium was reduced from 10 to 6 ml.

Source of ~~Erythrotin~~ Vitamin B_{12} inactivating factor: Aqueous extract of dried hog duodenal mucosa (Eli Lilly and Co.).

Micrograms of Mucosal Extract	Growth Response in Galvanometer Deflections Micrograms of ~~erythrotin~~ vitamin B_{12} per culture				
	0	0.3	1.0	3.0	10
0	11	39	37	42	40
3		9	36	38	39
10			9	36	37
30	11			11	38
100	15	14	14		16

A toxic effect of gastric juice upon the growth response of Lactobacillus lactis Dorner and Lactobacillus leichmannii had previously been observed, but due to the erratic nature of the response of these organisms,

the inactivation of vitamin B_{12} was not at first appreciated. Subsequently, however, the inactivation was demonstrated to occur in an identical fashion when these organisms are used.

The reaction is a highly specific one and only members of the vitamin B_{12} group are bound. The activities of other factors which can be substituted for this vitamin functionally, but which are chemically unrelated are unaffected. For example, the gastric principle does not alter the growth response produced by methionine in the Escherichia coli assay for vitamin B_{12} nor the response of purines, folic acid, and the desoxyribosides (165)(166)(167) in lactobacilli tests.

Erythein (signifying red protein) has been chosen as a suitable chemical name for the substance formed when erythrotin is bound by the protein in gastric juice. Apoerythein, then, designates the protein as it is secreted and as it occurs in the secreting mucosa prior to its combination with erythrotin.

After a partial characterization of the chemical properties of apoerythein had been made, we naturally were interested in determining what, if any, relationship existed between apoerythein and the intrinsic factor of Castle. A number of lines of evidence led us to postulate that apoerythein is the intrinsic factor, or an important component thereof. First, apoerythein has the chemical properties ascribed to the intrinsic factor. It is a protein, thermolabile, non-dialyzable, precipitated by alcohol, acetone, and ammonium sulfate, and inactivated by proteolytic enzymes. The following table summarizes typical results.

TABLE XV

CHEMICAL PROPERTIES OF APOERYTHEIN

	Vitamin B_{12} Erythrotin binding capacity millimicrograms per milliliter
Normal gastric juice	50
Normal gastric juice, heated (100°C. 15 minutes)	0
Normal gastric juice, dialyzed (24 hours)	ca. 40
Filtrate from normal gastric juice to which 4 volumes acetone had been added	0
Filtrate from normal gastric juice to which 4 volumes ethanol had been added	0
Filtrate from normal gastric juice 80% saturated with ammonium sulfate	0
Tryptic digest of normal gastric juice (24 hours)	0

Secondly, it was reasoned that in vivo apoerythein undoubtedly combines with the vitamin B_{12} components of the diet in a manner comparable to its behavior in the nutrient medium for bacteria; thus apoerythein must in some way affect the course of the vitamin during the digestive process.

A third, and more direct, method of correlating the activity of the physiological factor and this chemical substance was made by determining the relative concentrations of apoerythein in the gastric juice of normal patients and patients with pernicious anemia.

The apoerythein content of several samples collected from a normal subject at different times showed considerable variation. The easiest quantitative way to assess the importance of the combining capacity of the gastric juice is in terms of daily secretion. Assuming the average gastric secretion of a normal individual to be a liter and a half a day, we have calculated that, for normal subjects, at least 25 micrograms of vitamin B_{12} could be bound per day. In the anemic patients (with the exception of case V) less

TABLE XVI

ERYTHROTIN COMBINING CAPACITIES OF GASTRIC JUICE SAMPLES (31)

	Millimicrograms erythrotin bound per milliliter of secretion
Normal Subjects	
I (Author)	60
II * (32)	20
III (pooled samples from 3 subjects)* (32)	60
IV Specimen obtained from local hospital laboratory** (33)	15
Pernicious Anemia Patients	
I Local patient	>5
II (pooled samples from 3 subjects)*	>5
III * (32)	1
IV * (32)	1
V * (32)	>15

(32) * Lyophilyzed samples ~~obtained for us~~ furnished us through the courtesy of Dr. Edward Campbell of Eli Lilly and Co.

(33) ** I am indebted to Dr. Jerome Ravel for assistance in obtaining this specimen as well as for other specimens used in previous exploratory investigations not reported here.

than 1.5 micrograms can be bound during a twenty four hour interval.* Inasmuch as the daily requirement for vitamin B_{12} is considered to be of the order of 1 microgram per day (172), and considering the non-utilization of a considerable portion of the apoerythein due to its destruction by proteolytic enzymes and due to a failure of a considerable portion of it to contact vitamin B_{12} when the stomach is empty, it is obvious that the pernicious anemia patient will secrete an insufficient amount of apoerythein to bind an adequate amount of his dietary intake of vitamin B_{12} to meet his physiological requirement for this vitamin (if it must be so bound to be utilized). The normal subject, on the other hand, secretes a 15 to 20 fold excess of the apoerythein needed to bind the indicated minimum daily requirement of vitamin B_{12}.

In addition to the similarity of chemical properties, the specific nature of the combination, and the correlation between the apoerythein content of gastric juice and the anemic state, there is a fourth reason for postulating the identity of this protein factor with the physiological intrinsic factor of Castle. This fourth reason is based upon studies which have established the occurrence and distribution of the vitamin B_{12} binding factor by means of microbiological assays. Early clinical studies undertook to localize the secretion site of the intrinsic factor. These early experiments indicated that in swine the site of intrinsic factor secretion was the lower portion of the stomach and the duodenal portion of the intestine (3). Therefore, the gut of a freshly slaughtered hog was analyzed by microbiological techniques for the purpose of finding the distribution of apoerythein. The following table indicates that the protein distribution parallels that previously determined for the physiological factor by therapeutic trials.

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* Subsequent analyses by Mrs. Dunlap on additional control subjects and anemic patients were in line with the findings in the limited number of cases first tested (Table XVI).

TABLE XVII*

THE APOERYTHEIN CONTENT OF A HOG'S GASTRO-INTESTINAL TRACT

	Micrograms of erythrotin (vitamin B_{12}) bound per gram of moist tissue	
	Mucosal layer	Muscular layer
Stomach		
Cardiac region	0.12	0.06
Fundic region	0.67	0.11
Pyloric region	0.55	0.11
Intestine		
Duodenum	3.6	0.02
Jejunum	1.4	0.10
Ileum	0.16	0.04

* I am deeply indebted to Mrs. Wayne Dunlap for determining the distribution of apoerythein in the gastro-intestinal tract of the hog when illness prevented my carrying out this experiment.

Although these four lines of evidence just presented all point to the identity of the intrinsic factor and apoerythein, conclusive proof must await the demonstration that apoerythein possesses therapeutic activity when used in the oral treatment of pernicious anemia. The biochemical group of Eli Lilly and Company which have cooperated with us in supplying specimens are now sponsoring the clinical trial of concentrates of the protein.

The complex, erythein, parallels in many respects the behavior of the biotin-avidin combination. Erythein decomposes upon heating and liberates a substance which appears to have the same chemical and biological properties which were possessed by the erythrotin prior to its participation in the complex formation. Thus it possesses the microbiological activity of the original erythrotin, it can be combined with a fresh molecule of apoerythein, and on paper chromatographic analysis (using lutidine) it migrates at the same rate as erythrotin.

When normal gastric juice is used as the apoerythein source, the rate of destruction of apoerythein as measured by its erythrotin inactivating capacity or the rate of cleavage of the complex corresponds closely to the rate which has been observed for the inactivation of the intrinsic factor. However, the inactivation of the mucosal extract was found to vary to a degree considerably greater than had been anticipated. The presence of extraneous material appears to affect the rate at which the extract loses its erythrotin combining capacity, and heating for as long as 15 minutes at 120° C. may be necessary to completely decompose the complex or prevent the mucosa from inactivating any vitamin B_{12}.

In spite of the interesting phenomena disclosed by these investigations, it is not yet possible to explain the function of apoerythein nor the purpose of the extrinsic-intrinsic interaction. We postulated that through the complex formation with apoerythein, vitamin B_{12} was protected from destruction in the digestive tract. Experiments utilizing one sample of gastric juice did indicate that following apoerythein combination the vitamin B_{12} activity is less susceptible to destruction by mild alkaline conditions or dilute oxidizing agents. It was therefore postulated that a possible function of apoerythein might be the prevention of a comparable destruction in the digestive tract (237). Subsequent tests of this hypothesis by my colleagues using more refined preparations of apoerythein derived from hog mucosa and using a number of other samples of normal gastric juice indicate that this protective action usually cannot be demonstrated. The results obtained the first time this hypothesis was tested were probably the effect of other factors present in the particular gastric juice used, and consequently the activity of the intrinsic factor must be attributed to some other type of mechanism not yet understood.

SUMMARY

SUMMARY

In summary, it can be said that this problem has proven interesting from the standpoint of the opportunity it offered for the integration and evaluation of existing ideas, as well as the chance to develop and test hypotheses of our own.

From a practical standpoint, the final answer to the questions asked in the introduction to the experimental section have not been obtained. Certain possibilities have, however, been eliminated, and the probable identity of the extrinsic factor and a specific protein, apoerythein, has been established. The significant results reported here, are:

1. It was shown that the intrinsic factor activity of normal gastric juice can not be attributed to a counter inhibition of the agents in natural material which prevent the liberation of folic acid from its conjugates by conjugase.

2. It was shown that a xanthine or xanthopterin oxidase-like action upon folic acid can not account for the intrinsic factor activity of normal gastric juice.

3. It was shown that incubating sources of the extrinsic factor with intrinsic factor preparations caused no significant increase in microbiological activity when the mixtures were tested in a number of assays which responded to unidentified factors present in crude liver extracts.

4. Gastric juice was shown to contain a protein, which we have designated apoerythein, capable of forming with erythrotin (vitamin B_{12}) a thermolabile complex, erythein, in which form the vitamin is unavailable to those microorganisms which require it for growth. We have postulated the identity of apoerythein with the intrinsic factor of Castle or an important component thereof.

Four lines of evidence are presented which indicate the validity of this identity.

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